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The Metabolic Needs of Epithelial to Mesenchymal Transition

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Lista de Publicações / List of Publications

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Paper I – Joana B. Nunes, Joana Peixoto, Paula Soares, Valdemar Máximo, Sandra Carvalho, Salomé Pinho, André F. Vieira, Joana Paredes, A. C. Rego, Ildete L. Ferreira, Maria Gomez-Lazaro, Manuel Sobrinho-Simões, Keshav K. Singh, Jorge Lima. OXPHOS dysfunction regulates integrin-β1 modifications and enhances cell motility and migration. Hum Mol Genet. 2015 Apr 1;24(7):1977-90. doi: 10.1093/hmg/ddu612. Epub 2014 Dec 11.

Paper II – Joana B. Nunes, Michal Nagiec, Sejeong Shin, Sang-Oh Yoon, John M. Asara, Jorge Lima, John Blenis. UGP2 regulates ERK2-driven EMT in breast epithelial cells. *In preparation*

Os seguintes artigos não fazem parte do corpo principal de resultados desta tese, mas são parte integrante da mesma, tendo sido utilizados na sua Introdução e Discussão.

Annex I – Raquel G. Martins*, Joana B. Nunes*, Valdemar Máximo, Paula Soares, Joana Peixoto, Telmo Catarino, Teresa Rito, Pedro Soares, Luísa Pereira, Manuel Sobrinho-Simões, Ana Paula Santos, Joana Couto, Rui Henrique, Joana Matos-Loureiro, Paula Dias, Isabel Torres, Jorge Lima. 2013. A founder SDHB mutation in Portuguese paraganglioma patients. Endocr Relat Cancer. 2013 Nov 4;20(6):L23-6. doi: 10.1530/ERC-12-0399.

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Annex II – Joana B. Nunes, John Blenis, Jorge Lima. Fueling EMT: how do cancer cells shape their metabolism? *In preparation (review paper)*

Em cumprimento com o disposto no Decreto-Lei nº 388/70, declara que participou ativamente na recolha e estudo do material incluído e redigiu todos os trabalhos. Esta Dissertação inclui também resultados de trabalhos não publicados.

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Nota Explicativa

A presente dissertação encontra-se escrita em Inglês na sua quase totalidade, por ter sido co-orientada por um cientista norte-americano, e por se prever que alguns membros do júri sejam estrangeiros.

Para os meus avós.

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ABSTRACT

The study of cancer metabolism is at the forefront of cancer research today. Ever since the initial studies made by the Nobel laureate Otto Warburg, scientists have tried to understand how a cancer cell shapes its metabolism, in order to cope with stressful conditions and oncogenic demand. A key aspect in tumor development is the acquisition of migratory and invasive capabilities, which ultimately empower cancer cells with the ability to spread and create metastases in distant sites.
In this thesis, I will focus on studying the metabolism of cancer cells. Specifically, I will try to understand the metabolic processes that influence the capacity of cancer cells to invade adjacent tissues, which is the first step of metastasis formation.
In order to attain my objectives, I set out two questions that were specifically addressed in chapters 3.1 and 3.2 respectively:

- How does altered metabolism affect the cancer cell phenotype?
- How does a more aggressive cancer cell phenotype regulate metabolism?

For the first question, we generated an *in vitro* model comprised of cybrid cell lines, where we induced a metabolic injury –oxidative phosphorylation dysfunction – arising as a consequence of a mitochondrial DNA (mtDNA) mutation. In this model, by comparing wild-type against mutant mtDNA in the same nuclear background, we concluded that defective oxidative phosphorylation endows cancer cells an increased capacity to move and migrate, having also a positive impact on their capacity to form metastasis.
When addressing the second question, we took in consideration that, in epithelial-derived cancers, the formation of metastasis has been associated with transdifferentiation towards a mesenchymal phenotype, i.e. epithelial-to-mesenchymal transition (EMT). On this basis, we took advantage and optimized a model of EMT, where we characterized the metabolic changes that occur during this transition, in an attempt to identify the metabolic traits that are fundamental for the EMT-related migration and invasion. Our results showed an enrichment of the UDP-glucose pathway in cells undergoing EMT, namely high levels of UDP-glucose and upregulation of UGP2, the enzyme that catalyzes UDP-glucose production. We could further demonstrate that the expression of UGP2 is required for EMT in an ERK2-driven model. Finally, our results suggest that UGP2 might be modulating the production of hyaluronan, which may be a key modulator of EMT.
In summary, we believe that the works laid in this thesis provide new insights about the role played by altered metabolism in the oncogenic properties of cancer cells. Most importantly, they raise new questions and open avenues for research that can bring light into the determinants of cancer cells.

RESUMO

Atualmente, o estudo do metabolismo do cancro está na linha da frente da investigação em cancro. Desde os estudos iniciais do investigador laureado com o prémio Nobel, Otto Warburg, que os cientistas tentam perceber de que maneira a célula cancerígena adequa o seu metabolismo às condições de stress e sinais oncogénicos a que está sujeita. Um dos aspetos principais no desenvolvimento tumoral é a aquisição de capacidade migratória e invasiva, que permite às células cancerígenas disseminarem-se e formar metástases em órgãos distantes.

Nesta tese, irei focar-me no estudo do metabolismo das células cancerígenas. Em particular, vou tentar identificar os processos metabólicos que influenciam a capacidade das células cancerígenas de invadir os tecidos adjacentes, o primeiro passo na formação de metástases.

De modo a atingir os meus objetivos, formulei duas questões que são respondidas nos capítulos 3.1 e 3.2, respetivamente:

- Como é que o metabolismo alterado afeta o fenótipo da célula cancerígena?
- Como é que uma célula mais agressiva regula o seu metabolismo?

De maneira a responder à primeira questão, construímos um modelo *in vitro* de linhas células cibridas, onde gerámos um problema metabólico – disfunção na fosforilação oxidativa – em consequência de uma mutação no ADN mitocondrial. Neste modelo, comparando o ADN mitocondrial normal com o ADN mutado, perante o mesmo conteúdo nuclear, concluímos que uma fosforilação oxidativa deficiente leva a uma capacidade de movimento e migração aumentada e também tem um impacto positivo na capacidade de formar metástases.

Para responder à segunda questão, tivemos em consideração que, em cancros originados de tecidos epiteliais, a formação de metástases está frequentemente associada a uma transdiferenciação para um fenótipo mesenquimal, *i.e.* transição epitélio-mesenquimal (TEM). Nesta base, tirámos partido e otimizámos um modelo de TEM, em que caracterizamos as alterações metabólicas que ocorrem durante esta transição, de maneira a identificar traços metabólicos fundamentais para a migração e invasão associadas à TEM. Os nossos resultados mostraram um enriquecimento na via da UDP-glucose nas células em TEM, nomeadamente níveis elevados de UDP-glucose e uma sobreexpressão da UGP2, a enzima que catalisa a formação de UDP-glucose. Demonstrámos posteriormente que a expressão da UGP2 é necessária para a TEM num modelo onde este processo é induzido pela ERK2. Por fim, os nossos resultados sugerem que UGP2 intervêm na modelação da produção de ácido hialurónico, um mediador importante durante a TEM.

Concluindo, acreditamos que o trabalho desta tese providenciou novas perspetivas sobre o papel do metabolismo alterado nas propriedades oncogénicas das células cancerígenas. De salientar que

estas levantam novas questões e abrem novas perspetivas de investigação que podem trazer luz aos determinantes das células cancerígenas.

ABBREVIATIONS LIST

^{18}F-FDG: ^{18}F-fluorodeoxyglucose

2-DG: 2-deoxy-D-glucose

2-DG6P: 2-deoxy-D-glucose-6-phosphate

2-HG: 2-hydroxyglutarate

α-KG: α-ketoglutarate

α-SMA: α-smooth muscle actin

A: alanine

Acetyl CoA: acetyl coenzyme A

ADAM: a disintegrin and metalloprotease

ADP: adenosine diphosphate

ANT: ADP/ATP translocase

ATP: adenosine triphosphate

BCAR1: breast cancer anti-estrogen resistance 1

BME: basement membrane extract

BMP: bone morphogenetic protein

BRAF: B-Raf proto-oncogene, serine/threonine kinase

C-terminal: carboxyl-terminal

CAIX: carbonic anhydrase IX

CD: common docking

CDH1: cadherin 1

CDH2: cadherin 2

CDK4: cell division kinase-4

CDS: coding DNA sequence

COX: cytochrome c oxidase

CRC: colorectal cancer

CST: Cell Signaling Technologies

CytC: cytochrome c

D: aspartic acid or aspartate

ddH2O: double distilled water

DGL1: discs large MAGUK scaffold protein 1

DMEM: Dubelcco's modified Eagle medium

DMSO: dimethyl sulfoxide

DN: D319N (aspartate to asparagine in the amino acid position 319) mutation on ERK2

DNA: deoxyribonucleic acid

DOCK3: dedicator of cytokinesis 3

DOX: doxycyclin

ECL: enhanced chemiluminescence

ECM: extracellular matrix

EGF: epidermal growth factor

EGR1: early growth response protein 1

EMT: epithelial to mesenchymal transition

ERK: extracellular signal–regulated kinase

ESCR-III: endosomal sorting complexes required for transport III

ETC: electron transport chain

EZH2: enhancer of zeste homolog 2

F6P: fructose-6-phosphate

FAD: Flavin adenine dinucleotide, oxidized form

$FADH_2$: Flavin adenine dinucleotide, reduced form

FAK: Focal adhesion kinase

FBP: fructose-1,6-biphosphatase

FBS: fetal bovine serum

FGF: fibroblast growth factor

FH: fumarate hydratase

Fig.: figure

FSP-1: fibroblast-specific protein-1

G6P: glucose-6-phosphate

G1P: glucose-1-phosphate

GDH: glutamate dehydrogenase

GDP: guanosine diphosphate

GDT: guanosine triphosphate

GEF: guanine nucleotide exchange factor

GLS: glutaminase

GLUT: glucose transporter

GSK: glycogen synthase kinase

GTP: guanosine triphosphate

h: hours

H^+: protons

H_2O: water

HA: hyaluronan

HABP: HA binding protein

HAS2: hyaluronan synthase 2

HBP: hexosamine biosynthetic pathway

HCC: hepatocellular carcinoma

HEK: human embryonic kidney

HGF: hepatocyte growth factor

HIF-1: hypoxia-inducible factor 1

HKII: hexokinase II

HMGA2: high mobility group A2

HNF-1β: hepatocyte nuclear factor-1β

HOTAIR: HOX transcript antisense intergenic RNA

HRE: hypoxia-response element

IDH: isocitrate dehydrogenase

IDT: Integrated DNA Technologies

ILK: Integrin linked kinase

JNK: c-Jun N-terminal kinases

LC-MS/MS: liquid chromatography-tandem mass spectrometry

MAPK: mitogen-activated protein kinase

min: minutes

miR: micro RNA

MMP: matrix metalloproteinase

mtDNA: mitochondrial DNA

N: asparagine

NAD^+: nicotinamide adenine dinucleotide, oxidized form

NADH: nicotinamide adenine dinucleotide, reduced form

NAPDH: Nicotinamide adenine dinucleotide phosphate, reduced form

ND: NADH dehydrogenase

NEDD9: neural precursor cell expressed, developmentally down-regulated 9

NF-kB: nuclear factor kappa B subunit 1

NRF2: nuclear factor erythroid-2–related factor 2

NSCLC: non-small cell lung cancer

N-terminal: amino-terminal

o/n: over-night

OAA: oxaloacetate

ºC: degrees Celsius

O-GlcNAc: O-linked β-N-acetylglucosamine

OGT: O-linked β-N-acetylglucosamine transferase

O_2: oxygen

OXPHOS: oxidative phosphorylation

Par: partitioning defective

PAS: periodic acid–Schiff

PBS: phosphate buffer saline

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PDAC: pancreatic ductal adenocarcinoma

PDGF: platelet-derived growth factor

PDH: pyruvate dehydrogenase

PET: positron emission tomography

PFK: phosphofructokinase

PFKFB4: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4

PGC-1α: peroxisome proliferator-activated receptor-γ co-activator 1α

PHA-E: *Phaseolus vulgaris* erythroagglutinin

PHA-L: *Phaseolus vulgaris* leucoagglutinin

PHD: prolyl hydroxylase domain enzymes

PHGDH: 3-phosphoglycerate dehydrogenase

PI3K: phosphatidylinositol 3-kinase

PK: pyruvate kinase

PPP: pentose phosphate pathway

PRC: polycomb repressive complex

Prrx1: paired-related homeobox transcription factor 1

PTEN: phosphatase and tensin homolog

qPCR: quantitative PCR

Rb: retinoblastoma protein

RHAMM: receptor for hyaluronic acid-mediated motility

RNA: ribonucleic acid

ROS: reactive oxygen species

rpm: rotations per minute

rRNA: ribosomal RNA

RSK: p90 ribosomal S6 kinase

RT: room temperature

SCBT: Santa Cruz Biotechnology

SCRIB: scribbled planar cell polarity protein

SD: standard deviation

SDH: succinate dehydrogenase

SDS: sodium dodecyl sulfate

TBP: TATA box binding protein

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline with 0.5% Tween

TCA cycle: tricarboxylic cycle

TET: ten-eleven-translocation

Tet: tetracycline

TFs: transcription factors

TGF-β: transforming growth factor-β

TIC: total ion chromatogram

TIGAR: TP53-induced glycolysis and apoptosis regulator

TNF-α: tumor necrosis factor-α

tRNA: transfer RNA

UAP1: UDP-N-acetylglucosamine pyrophosphorylase 1

UDP: uridine diphosphate

UDP-GlcNAc: UDP-N-acetylglucosamine

UGDH: UDP-glucose 6-dehydrogenase

UGP2: UDP-glucose pyrophosphorylase 2

VDAC: voltage-dependent anion channel

VEGF: vascular endothelial growth factor

VHL: von Hippel-Lindau

WASF2: WAS protein family member 2

WT: wild-type

Y: tyrosine

YA: Y261A (tyrosine to alanine in the amino acid position 261) mutation on ERK2

ZEB: zinc finger E-box binding homeobox

ZO: zonula occludens

ZONAB: ZO-1-associated nucleic acid-binding protein

PROLOGUE

According to the World Health Organization, cancer is the second main cause of death worldwide and the number of people affected by this disease is expected to increase by 70% in 20 years. The most lethal types of cancer are those of the lung, liver, colon/rectum, stomach and breast (World Health Organization 2018).

Cancer is a generic term for a large group of diseases, characterized by an abnormal cell growth that occurs as consequence of genetic alterations and environmental deregulation of homeostasis and that does not respect the tissue borders and spreads. Cancers can be divided, according to the tissue of origin, into several major subtypes: carcinomas (epithelial tissue), sarcomas (connective tissue), lymphomas (lymphatic system), leukemias (bone marrow), myelomas (plasma cells of bone marrow) and mixed types (World Health Organization 2013). Metastasis is the major cause of cancer-related deaths (World Health Organization 2018). In epithelial-derived cancers, metastasis constitutes a multi-step cascade that includes the detachment of the primary tumor cells and invasion of the surrounding tissue, the intravasation into the circulatory system and the formation of a secondary tumor colony in a lymph node or at a distant site. In the case of distant metastasis, cells traveling in the bloodstream have to extravasate through the vascular walls to form a new tumor. Several studies have demonstrated that carcinoma cells that locate at the invasive front of primary tumors acquire a mesenchymal phenotype and that epithelial to mesenchymal transition (EMT) is an important process enabling metastasis (Thiery et al. 2009) (Fig. 1).

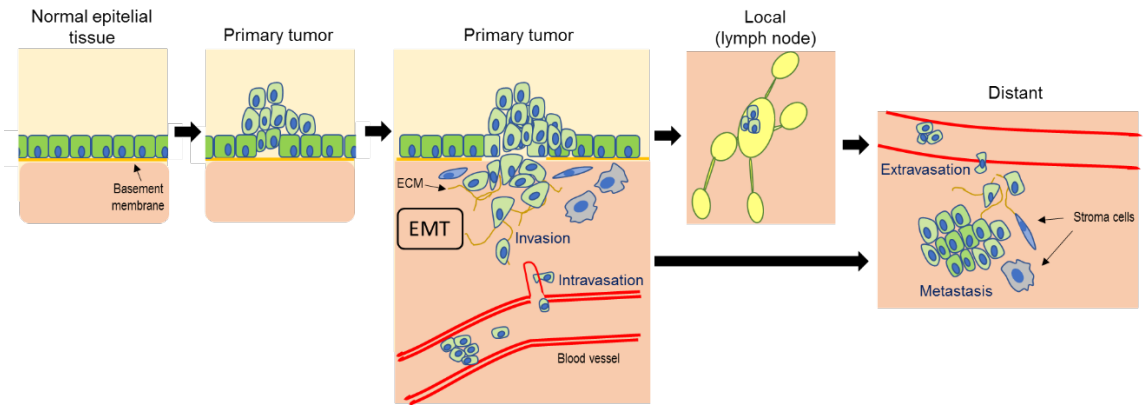

Fig. 1: Process of metastasis. EMT, epithelial to mesenchymal transition; ECM, extracellular matrix.

The present thesis will focus on cancer metabolism, as a crucial aspect of tumorigenesis and cancer progression. Chapter 1.1 will describe the alterations in metabolism that characterize cancer cells. In this thesis, I was particularly interested in the metabolic alterations during metastasis formation, more specifically in its first step when cancer cells migrate and invade the surrounding tissue of the primary tumor (chapter 1.2) and where EMT is one of the cellular programs that empowers cancer cells with migratory capacity (chapter 1.3). The results section of this thesis contains two studies on cancer metabolism (in a scientific manuscript format) aiming at answering the following questions: what are the consequences for cancer cell behavior when the cellular metabolism is modulated? (chapter 3.1) and what are the metabolic alterations upon the acquisition of a more aggressive phenotype? (chapter 3.2). Finally, chapter 4 contains a general discussion of both studies. Two papers that were used for the introduction and discussion of this thesis, and where I am first author, are attached at the end of the thesis (annexes I and II). One relates to the screening of mutations in metabolic enzymes (succinate dehydrogenase) in Portuguese paraganglioma patients, where we discovered a new succinate dehydrogenase B founder mutation. The other is a thorough literature review about the metabolism that characterizes EMT.

1 - INTRODUCTION

1.1 - CANCER METABOLISM

1.1.1 - Cancer metabolism – overview

The metabolism of tumors has been subject of study since the beginning of the twentieth century. The pioneer in the field, Otto Warburg, described a high consumption of glucose and release of lactate by malignant cells, when compared with non-neoplastic cells, even in presence of oxygen (Warburg 1924); this phenomenon is known as aerobic glycolysis or the "Warburg effect". Warburg hypothesized that this aerobic glycolysis was irreversible and due to an impairment of mitochondrial respiration (Warburg 1956). Nowadays, with an exponential interest in this field of research and the inclusion of the "reprogramming of energy metabolism" as a hallmark of cancer (Hanahan and Weinberg 2011), a more complex picture has emerged.

The adaptation of the cellular metabolism is very important in the sense that it allows cells to maintain cellular homeostasis and survive under different environmental conditions, being part of the adjustment to a phenotype alteration. Therefore, during malignant transformation and tumor progression, cancer cells rely on specific changes in metabolic activity in order to meet their energetic demands and biomass production. Nowadays, we know that these changes are not limited to carbohydrates metabolism but they also include lipids, nucleotide, amino acid, as well as alterations in reductive equivalents and redox power. Besides, different types of tumors display different metabolic adaptations and even within the tumor microenvironment there is a metabolic cooperation between cancer cells and the cells that constitute the tumor stroma. The role of metabolism in tumorigenesis is also linked to other aspects of a cancer such as stemness, exosomes, angiogenesis, immune escape, among other. Importantly, cancer cell metabolism reflects the stage of the tumor, implying that it may vary according to the proliferation rate, and to the migratory and invasive capacities of cancer cells. Indeed, much less is known about the metabolism of migratory cancer cells and of those that are detaching from the primary tumor, as the first step of secondary tumor sites colonization.

1.1.2 - Once upon a time, there was a scientist named Warburg

In the first half of the twentieth century, the German scientist Otto Warburg described a phenomenon that would alert the scientific community for the metabolism of tumors (Warburg 1924). Measuring oxygen consumption and lactate production simultaneously in tumor slices, Warburg reported that, even in the presence of oxygen, proliferating tumor cells consumed glucose at a much higher rate when compared with normal cells and secreted most of the glucose-derived carbon in the form of lactate (Warburg 1924; Warburg and Minami 1923). A century ago,

Louis Pasteur had already demonstrated that fermentation was reduced by the presence of oxygen, being pyruvate conducted to the mitochondria to be oxidized (Racker 1974). The "Pasteur effect" reflects the versatility and dynamic nature of metabolism which, in this case, plays an important role in maintaining energy production throughout a range of oxygen concentrations in mammalian cells (Gatenby and Gillies 2004). However, Warburg pointed out that, in the case of tumor cells, this increased glycolysis happens even in aerobic conditions. Warburg proposed that this "aerobic glycolysis" was due to an impairment in respiration and it was a driving force of tumorigenesis (Warburg 1956).

After a period with declining interest on the metabolism of cancer cells, where cancer research focused on the genetic basis of the disease, a new imaging technique based on Warburg's principle brought it up to the stage again. PET (positron emission tomography) is based on the detection of photons released by annihilation of positrons emitted by radiopharmaceuticals. ^{18}F-fluorodeoxyglucose (^{18}F-FDG), the most commonly used PET tracer, is a glucose analogue whose use has shown that most primary and metastatic human cancers have a significantly increased glucose uptake (Kelloff et al. 2005; Som et al. 1980). The success of this technique in the detection, classification, staging and therapeutic management of tumors was actually one of the reasons for the reemergence of the interest for tumor metabolism. Besides, the discovery of oncogenic mutations in mitochondrial metabolic enzymes and the association of the "Warburg effect" with mutations in "classical" oncogenes and tumor suppressor genes, boosted not only the exploration of the mitochondrial function in tumor cells but also travelling beyond the bioenergetics point of view to the general metabolism of tumor cells. In fact, in 2011, the reprogramming of energy metabolism was considered as one of the "emerging" common traits of cancer cells (Hanahan and Weinberg 2011).

1.1.3 - Cancer metabolism – what do we know today?

Nowadays, we realize that cancer metabolism is incredibly complex. According to Hanahan and Weinberg, carcinogenesis is associated with self-sufficiency in growth signals, insensitivity to growth-inhibitory signals and limitless replicative potential (Hanahan and Weinberg 2000). These characteristics must be tightly associated with a metabolic reprogramming, since cells require biosynthetic precursors as building blocks to fuel cell growth. So, in the recent years cancer metabolism has been heavily studied under the perspective of its role in supporting the increased anabolic needs of enhanced proliferation (Vander Heiden, Cantley, and Thompson 2009).

In mammalian cells, the uptake of nutrients is controlled by growth factor signaling; however, cancer cells accumulate oncogenic alterations that lead to aberrantly activated signaling pathways, providing them some independence from these external requirements (Hanahan and

Weinberg 2000; Thompson 2011). Indeed, the reprogramming of tumor metabolism has been shown to be under the control of various oncogenic signals (Edinger and Thompson 2002; Levine and Puzio-Kuter 2010; Thompson 2011). Oncogenic *KRas*, for example, enhances glycolysis in several cell types and, in the case of pancreatic ductal adenocarcinoma (PDAC), it diverts the glycolytic flux towards anabolic pathways, such as the hexosamine biosynthetic pathway (HBP) for protein glycosylation and pentose phosphate pathway (PPP) for ribose production (Racker, Resnick, and Feldman 1985; Ying et al. 2012; Yun et al. 2009). In mutant *KRas, p53* null lung tumors, glucose metabolism is also rewired in low- and high-grade lesions and modulated by mutant *KRas* allelic content. In fact, a higher mutant *KRas* (G12D) copy number is associated with a glycolytic phenotype and with an increased flux of glucose metabolism towards tricarboxylic (TCA) cycle and glutathione synthesis. Besides, the *KRas* mutant high-grade lung tumors showed a high sensitivity to combined glucose and glutathione depletion (Kerr et al. 2016). Conversely, recent evidence in yeast shows that, Ras is activated by a high glycolytic rate through one of its intermediates, fructose 1,6-bisphosphate, suggesting a reciprocal stimulatory signaling loop between cell proliferation and glycolysis (Peeters et al. 2017).

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling is another pathway that modulates cellular metabolism. Akt activity can induce glucose transporter (GLUT) 1 expression and trafficking, increase glycolytic rates and promote glucose consumption and lactate production. Moreover, its pro-survival role requires the presence of glucose in the culture medium, the induction of glycolysis and maintenance of a physiologic mitochondrial potential (Barthel et al. 1999; Plas et al. 2001; Rathmell et al. 2003).

The oncogene *c-Myc* stimulates glutamine utilization through the coordinate expression of genes necessary for cells to engage in glutamine catabolism (Wise et al. 2008). For example, c-Myc can upregulate glutaminase (GLS) by regulating miR-23a and miR-23b, being glutamine and GLS required for Myc-mediated cancer cell proliferation and survival (Gao et al. 2009). The translation efficiency of *c-Myc* is, in turn, regulated by mTOR/S6K1 signaling, via eIF4B, leading to a mTOR-dependent control of the glutamine flux through regulation of GLS (Csibi et al. 2014). mTOR, a signaling pathway implicated in cancer, can indeed regulate metabolism in multiple ways (Gomes and Blenis 2015). For example, mTORC1, through S6K1 or ATF4 can also induce pyrimidine and purine synthesis, respectively, thus fueling nucleic acid biosynthesis and promoting anabolic growth (Ben-Sahra et al. 2013, 2016; Robitaille et al. 2013).

p53 has also been shown to regulate metabolism (Flöter, Kaymak, and Schulze 2017), for example through the induction of TP53-induced glycolysis and apoptosis regulator (TIGAR) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 (PFKFB4) (Bensaad et al. 2006; Ros et al. 2017). TIGAR is a fructose-2,6-bisphosphatase domain containing protein, which leads to a

decrease of the glycolytic byproduct fructose-2,6-bisphosphate - a phosphofructokinase-1 activator - increasing substrate delivery to the oxidative PPP. p53-induced TIGAR expression seems to be important to reduce the levels of reactive oxygen species (ROS) after exposure to DNA damaging agents (Bensaad et al. 2006). In cancer cells, p53 represses the transcription of PFKFB4, an enzyme that also regulates the concentration of the fructose-2,6-bisphosphate. Interestingly, loss of p53 function in cancer cells leads to PFKFB4 upregulation and, in this context, the enzyme is working as a phosphatase, negatively regulating fructose-2,6-biphosphate levels. In p53-deficient cancer cells, PFKFB4 is regulating PPP and NAPDH levels and is essential for the viability of these cells (Ros et al. 2017). Besides, *p53* wild-type (and not the *p53* mutant V122A) inhibits the PPP, through inhibition of glucose-6-phosphate dehydrogenase (G6PD) (Jiang et al. 2011).

It is thus clear that the genetic alterations characteristics of cancer cells are able to shape the metabolism of cells. This is epitomized in certain tumors, such as hereditary paragangliomas/phaeochromocytomas (associated with succinate dehydrogenase [*SDH*] mutations) and malignant gliomas (associated with isocitrate dehydrogenase [*IDH*] mutations), where mutations in metabolic enzymes are crucial elements to drive tumorigenesis and can create metabolic vulnerabilities (Gottlieb and Tomlinson 2005).

On the other hand, metabolic plasticity governs the ability of cells to survive the environment where they are inserted in, indicating that the metabolism of cancer cells is also tailored according to extrinsic factors, like nutrient and oxygen availability, interaction with the cellular and non-cellular stromal components.

While the study of the metabolic rewiring of cancer cells has provided major breakthroughs, most of the research concerns the link between the metabolic alterations of cancer cells and their proliferative capacity, while much less is known about the metabolism of cancer cells in the metastasis process.

The next sub-sections describe in more detail the most relevant metabolic phenotypes within the context of this thesis.

Glycolytic phenotype

Glycolysis

Glycolysis is a central metabolic pathway of the cell, which occurs in the cytoplasm. The catabolism of glucose into pyruvate produces intermediate metabolites for several anabolic

reactions, generating also 2 molecules of ATP (adenosine triphosphate) and 2 NADH (the reduced form of nicotinamide adenine dinucleotide) per molecule of glucose. The glycolytic pyruvate can be further oxidized in the mitochondria in the TCA cycle where ATP production is maximized. Alternatively, pyruvate can be converted into lactate in the cytoplasm by lactate dehydrogenase (LDH), with the regeneration of NAD^+ (the oxidized form of nicotinamide adenine dinucleotide) from NADH.

Glucose is imported into the cell by GLUTs. After entering the cells, glucose is immediately phosphorylated into glucose 6-phosphate via phosphate transfer from ATP, by hexokinase (HK). This irreversible first step of the glycolytic pathway reaction is very important because it prevents glucose from exiting the cell, promoting the formation of a concentration gradient that facilitates glucose uptake and allowing the utilization of glucose by several major pathways. In some contexts, isoforms I and II of HK can physically and functionally interact with the mitochondria, promoting the 'open' configuration of the voltage-dependent anion channel (VDAC) that enables anionic metabolite exchange across the outer mitochondrial membrane. This is thought to allow a preferred access of HK to ATP synthesized in the mitochondria via the mitochondrial adenine nucleotide translocator; conversely, the ADP (adenosine diphosphate) generated by HK can be channeled back into the mitochondrion to support oxidative phosphorylation (OXPHOS) (Robey and Hay 2006). Glucose-6-phosphate is metabolized through a series of steps to generate pyruvate (Fig. 2). Another rate-controlling enzyme of glycolysis is phosphofructokinase (PFK) that exhibits a complex regulatory behavior, reflecting its ability to integrate many different signals from different pathways. PFK catalyzes the transfer of γ-phosphate from ATP to fructose-6-phosphate, generating fructose-1,6-bisphosphate and ADP. The reverse reaction is catalyzed by the rate limiting reaction of gluconeogenesis pathway, fructose-1,6-biphosphatase (FBP). Pyruvate kinase (PK) is also a major regulatory enzyme of glycolysis.

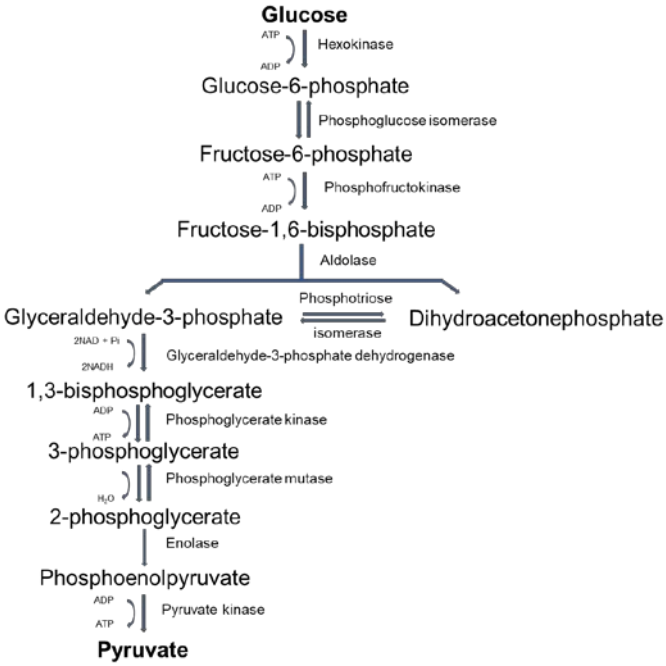

Fig. 2: Glycolysis.

The metabolism of glucose (and glutamine) is important to provide the reducing power, either in the form of NADH or $FADH_2$ (reduced form of flavin adenine dinucleotide), in this way driving ATP production through OXPHOS, which is essential for several anabolic reactions and for maintaining the redox capacity of cells. Besides, glucose (and glutamine) oxidation also produces NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate), which provides reducing power for a wide variety of biosynthetic reactions and helps maintaining the cellular redox capacity.

Aerobic glycolysis in cancer cells

The preference of cancers for aerobic glycolysis over OXPHOS has triggered an enormous interest. Since this question has been addressed from different angles, it increased our understanding of the role of metabolism in tumorigenesis and exposed its plastic nature.

As previously mentioned, the fact that tumors show an increase in glucose uptake has been confirmed by the successful use of ^{18}F-FDG PET, which has become a clinical valuable tool (Kelloff et al. 2005). In fact, glucose uptake is positively correlated with increased tumor aggressiveness and worse prognosis (defined by invasion, tumor size and metastasis) in gastric cancer (Mochiki et al. 2004), breast cancer (Alberini et al. 2009), oesophageal cancer (Kato et al. 2005), oral squamous cell carcinoma (Kunkel et al. 2003), soft tissue sarcomas (Schwarzbach et

al. 2005), among others. Besides, genes involved in glycolysis are overexpressed several human cancers (Altenberg and Greulich 2004). The advances in technology for the analysis of metabolic fluxes *in situ* revealed that increased glycolysis and lactate production is a feature of non-small cell lung cancer (NSCLC) (Davidson et al. 2016; Fan et al. 2009; Hensley et al. 2016). This was also shown using flux analysis in oncogenic PDAC tumor cells (Ying et al. 2012).

While the preference for a glycolytic metabolism over a more energy-profiting respiration can be regarded as an energetic constraint, cancer cell proliferation does not seem to be limited by ATP production (Lunt and Vander Heiden 2011). Although a fast glycolytic rate can supply high amounts of ATP (Pfeiffer, Schuster, and Bonhoeffer 2001), in fact glycolysis does not seem the major contributor of ATP in several cancer cells (Zu and Guppy 2004). Moreover, other proliferating cells, besides cancer cells, use aerobic glycolysis regardless of the nutrient and oxygen availability (Vander Heiden, Cantley, and Thompson 2009).

Why increased glycolysis? Much attention has been paid to the benefits of increased glycolysis in the context of the tumor microenvironment, especially during restriction in oxygen availability. The growth of the tumor mass and the fact that the microenvironment of a solid tumor generally has a disorganized microvasculature, creates regions of hypoxia (Carmeliet and Jain 2000; Helmlinger et al. 1997). Besides, fluctuations in tumor microvessel flow rate can also generate intermittent or perfusion-limited hypoxia (Kimura et al. 1996). Therefore, several authors have proposed that the glycolytic phenotype and a lower dependence on aerobic respiration confers a selective advantage for survival and proliferation during somatic evolution of cancer, within the unique tumor microenvironment (Gatenby and Gillies 2004; Hsu and Sabatini 2008). One of the most important modulators of the cellular transcriptional response to hypoxia and regulator of cellular metabolism is the hypoxia-inducible factor 1 (HIF-1). HIF-1 is a transcription factor first described by Semenza & Wang (Semenza and Wang 1992) and constitutes one of three mammalian HIF isoforms (Kaelin Jr. and Ratcliffe 2008). It binds to hypoxia-responsive elements (HRE) upon formation of a heterodimer composed by the constitutive β subunit (also known as aryl hydrocarbon receptor nuclear translocator) and the α subunit. A crucial mechanism involved in oxygen regulation of HIF activity is mediated by prolyl hydroxylase domain enzymes (PHD) and the E3 ubiquitin ligase, von Hippel-Lindau (VHL). Under normoxia, HIF-1α is targeted for proteasomal degradation by VHL, in consequence of its oxygen-dependent proline hydroxylation catalyzed by PHD. In low oxygen tension conditions, prolyl hydroxylation of HIF-1α is impaired, VHL cannot bind to the newly synthesized subunit, resulting in HIF-1α stabilization and accumulation in the cytosol. Eventually this subunit is translocated to the nucleus where it binds to the constitutively active HIF-1β subunit to form an active transcription factor (Kaelin Jr. and Ratcliffe 2008). HIF-1α can also be stabilized by numerous molecular players of signaling pathways dysregulated in cancer, in an oxygen independent manner (Gatenby and Gillies, 2004).

HIF is thought to play a role in the development of cancers from the von-Hippel Lindau spectrum, which are characterized by *VHL* loss-of-function mutations, as well as those developing upon *SDH* or fumarate hydratase (*FH*) mutations (Gottlieb and Tomlinson 2005). In the these, mitochondrial metabolism appears to be involved in HIF regulation during normoxia, through the generation of Krebs cycle intermediates such as succinate, fumarate and α-ketoglutarate (α-KG) (Pollard et al. 2005; Selak et al. 2005) and possibly ROS, although still controversial (Iommarini et al. 2017).

HIF-1 is important for promoting a shift towards certain forms of carbon metabolism and ATP production such as glycolysis, by regulating the transcription of metabolic genes such as the glycolytic enzymes *HKI*, *HKII* and *PFK*, besides *GLUT1* and *LDHA* (Hu et al. 2003; Semenza 2003).

Finally, it has been argued that suppression of OXPHOS protects cells from production of cytotoxic levels of ROS caused by hypoxia, besides preserving oxygen for other oxygen-mediated reactions and to avoiding necrotic death (Frezza and Gottlieb 2009).

Another component of the "Warburg effect" is the increased secretion of lactate, which can be produced from pyruvate through the action of LDH. LDH oxidizes NADH to NAD^+, contributing to replenishing the NAD^+ pools to sustain glycolysis. Moreover, it has been proposed that proliferating cancer cells tend to shuttle pyruvate into the production of lactate to avoid the negative regulation by high levels of ATP generated by the OXPHOS chain, if pyruvate integrates the TCA cycle (Pavlova and Thompson 2016). Lactate can also conduct an HIF-independent hypoxic response that promotes angiogenesis and tumor cell growth. This is mediated by NDRG, an oxygen-regulated protein and a substrate of the PHD2/VHL system, whose binding to lactate allows its accumulation during hypoxia, leading to the activation of the Raf- extracellular signal–regulated kinase (ERK) pathway and the expression of angiogenic marker genes (Lee et al. 2015). HIF-1 (and other oncogenes like c-Myc) are able to induce LDHA expression, thus acting as cellular hubs that connect the oncogenic signaling pathways with the metabolic pathways (Firth, Ebert, and Ratcliffe 1995; Semenza et al. 1996; Shim et al. 1997).

One of the consequences of the increased production of lactate by tumor cells is the reduction of intracellular pH, which is controlled by intracellular pH regulating systems such as cell membrane proton exchangers. In fact, cancer overexpress several of these acid extruders, like H^+-ATPases, the Na^+–H^+ exchanger and the bidirectional lactate/H^+ symporters monocarboxylate transporters 1 and 4 and it has been suggested that a higher intracellular pH in cancer cells may be permissive for tumorigenesis (Webb et al. 2011), since the inhibition of these adaptive mechanisms can impair tumor growth (Parks et al. 2017). Interestingly, an acid pH also induces the production of L-2-hydroxyglutarate (L-2-HG) (the D-2-HG enantiomer) which leads to the stabilization of HIF,

even in normoxic conditions. In these conditions, the production of this metabolite is catalyzed by LDHA which preferentially reduces α-KG at lower pH (Intlekofer et al. 2017).

After being produced, lactate is secreted and, therefore, increased lactate production contributes to extracellular acidosis. Extracellular lactate signals for angiogenesis, by inducing vascular endothelial growth factor (VEGF) expression (Fukumura et al. 2001; Xu, Fukumura, and Jain 2002) and acts as a cancer cell metabolic fuel (Kennedy et al. 2013; Sonveaux et al. 2008). Besides, acidosis promotes extracellular matrix (ECM) remodeling and stimulates acid-activated proteases to facilitate tumor cell invasion and dissemination (Busco et al. 2010; Kato et al. 1992; Rozhin et al. 1994). Lactate also affects non-tumor cells from the microenvironment, which will be discussed later in this chapter, in the section "Metabolism of tumors: much more than cancer cells".

Acidosis can also be promoted by the mitochondrial metabolism, through the formation of carbon dioxide. This is due to the release of carbon dioxide and generation of protons and bicarbonate by a class of extracellular carbonic anhydrases (Swietach, Vaughan-Jones, and Harris 2007). Carbonic anhydrase IX (CAIX) expression is increased in tumors (Swietach, Vaughan-Jones, and Harris 2007) and its activity is important for tumor cell survival, controlling the intracellular pH (Chiche et al. 2009). In turn, hypoxia also activates the HIF-1 pathway, which upregulates CAIX and leads to additional exacerbation of tumor acidosis (Dayan et al. 2006).

Although in proliferating cancer cells the glucose-derived lactate has important oncogenic effects, a significant fraction of glucose carbon, in the form of glycolytic intermediates, is shunted into multiple biosynthetic pathways instead of giving rise to pyruvate. Research has shown that these glycolytic intermediates provide essential anabolic support for cell proliferation and tumor growth.

Other destinies for glucose (anabolism)

Glucose is one of the most abundant nutrients in plasma and its major role in cellular metabolism is illustrated by the fact that it is the most well-studied metabolite concerning cancer. However, glucose or, more specifically, its phosphorylated form - glucose-6-phosphate - can be metabolized in many different pathways in the cell, in order to support the production of intermediates for the synthesis of lipids, proteins and nucleic acids. Here, I will briefly describe the most relevant pathways in cancer and for this thesis: PPP, serine synthesis, HBP and uridine diphosphate (UDP)-glucose pathway (Fig. 3).

Pyruvate kinase (PK) is a highly regulated enzyme that has a crucial role in balancing the outputs of glycolysis, having been proposed to favor this anabolic metabolism. This enzyme catalyzes the

last step of glycolysis by converting phosphoenolpyruvate into pyruvate, generating ATP. There are several isoforms of PK and some types of cancer express the M2 splice isoform, a less active form of the enzyme (Christofk, Vander Heiden, Harris, et al. 2008). The expression of PKM2 in cancer cells is associated with the aerobic glycolysis (Christofk, Vander Heiden, Harris, et al. 2008). Although a lower PK activity seems counter-intuitive, it has been proposed that PKM2 expression promotes anabolic processes since it causes a build-up of glycolytic intermediates, which is important for a proliferating cancer cell. For example, a reduced PK activity can promote serine biosynthesis and support proliferation in conditions where serine is limiting (Chaneton et al. 2012). However, this concept is still matter of debate and PKM2 seems not to be essential in some cancer types such as in mouse breast cancer or *Apc* loss-driven colon cancer models (Israelsen et al. 2013; Lau et al. 2017). Indeed, *PKM2* null mice are viable and develop spontaneous hepatocellular carcinomas (HCC) (Dayton et al. 2016). Interestingly, PKM2 also integrates signaling inputs, since its activity can be regulated not only by metabolites such as the allosteric activator fructose-1,6-bisphosphate and serine (Chaneton et al. 2012; Jurica et al. 2018), but also by tyrosine kinase signaling, which phosphorylates and inhibits the formation of the active tetrameric PKM2 (Christofk, Vander Heiden, Wu, et al. 2008; Hitosugi et al. 2009).

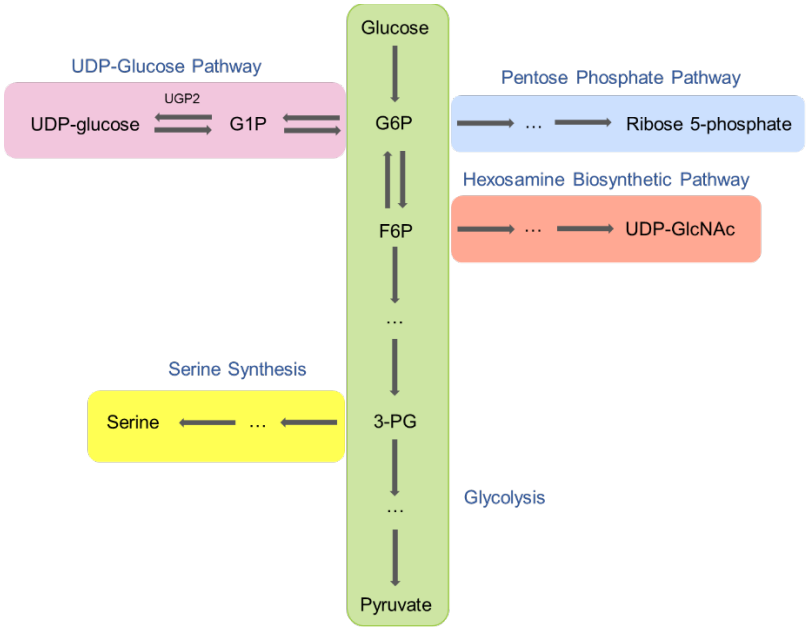

Fig. 3: Glucose fates in the cells: glycolysis, PPP, HBP, serine synthesis and UDP-glucose. Ellipsis (and the arrows before and after) represent multiple reactions. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 3-PG, 3-phosphoglycerate; G1P, glucose-1-phosphate; UDP-GlcNAc, UDP-N-acetylglucosamine; UGP2, UDP-glucose pyrophosphorylase 2.

Pentose phosphate pathway (PPP) and serine synthesis

Glucose-6-phosphate can be diverted to the PPP, which converts glycolytic intermediates into ribose-5-phosphate, in this way generating reducing equivalents and intermediates important for the synthesis of lipids and nucleotides and for redox control. The PPP is thus crucial in sustaining cell survival and proliferation. The PPP has two phases: the oxidative, which is the major source of NAPDH and the non-oxidative that does not produce NADPH (Fan et al. 2014; Stincone et al. 2015). The PPP is frequently altered in the setting of cancer. For example, the first and the rate limiting enzyme G6PD is inhibited by wild-type p53, which can result in PPP increased flux in cancer, since *p53* is known to be one of the most altered genes in cancer (Jiang et al. 2011). In the context of PDAC, there seems to be a decoupling between the oxidative and non-oxidative phases, and an increased glucose flux towards the non-oxidative PPP, possibly to sustain ribose biogenesis (Ying et al. 2012). Moreover, the expression of the PPP enzyme transaldolase is positively correlated with hepatocellular carcinoma progression and metastasis and it has been considered a biomarker since it can be detected in patients serum (Wang et al. 2011).

The amino acid serine can be synthetized *de novo* using the glycolytic intermediate 3-phosphoglycerate, via three sequential enzymatic reactions, the first of which is catalyzed by the NAD^+-dependent enzyme 3-phosphoglycerate dehydrogenase (PHGDH). This pathway also produces α-KG, which is important for TCA cycle anaplerosis of glutamine-derived carbon and confers the ability to cells to grow in the absence of extracellular serine (Possemato et al. 2011). *De novo* serine synthesis has been shown to be altered in cancer, for example through PHGDH copy number gain (Mullarky et al. 2011; Possemato et al. 2011) or through activation of a transcriptional program dependent on the transcription factor NRF2 (nuclear factor erythroid-2–related factor 2) (DeNicola et al. 2015). Serine is an important amino acid for the synthesis of purines and thymidine, since it can produce one carbon units, through the folate metabolism, either in the mitochondria or in the cytosol. Tumors cells can rewire the folate pathway to meet high proliferation demands in nutrient-replete conditions (Ducker et al. 2016). The mitochondrial folate-mediated NADPH production also has a role in controlling oxidative stress that may limit melanoma metastasis (Piskounova et al. 2015). The importance of serine and glycine for proliferative metabolism has been recently highlighted in a study that shows that dietary serine and glycine removal was sufficient to reduce tumor growth and increase survival in intestinal cancer (driven by *Apc* inactivation) or lymphoma (driven by *Myc* activation) (Maddocks et al. 2017).

Hexosamine biosynthetic pathway (HBP)

Fructose-6-phosphate can also be used in HBP, which comprises the series of metabolic reactions that produce UDP-N-acetylglucosamine (UDP-GlcNAC), which is a substrate for O-glycosylation, N-glycosylation and O-GlcNAcylation and for the formation of glycosaminoglycans and proteoglycans (Pinho and Reis 2015). HBP pathway receives input from glucose, amino acids (namely glutamine), nucleotides and fatty acid metabolic pathways, thus it can be considered an efficient nutrient sensor. Nutrient depletion, namely glucose or amino acid depletion can induce O-GlcNAcylation through an increase in glutamine-fructose-6-phosphate transaminase 1, the first and the rate-limiting enzyme of the HBP (Chaveroux et al. 2016).

The HBP was shown to be altered in cancer, as evidenced in PDAC, where oncogenic *KRas* redirects glucose metabolism toward HBP (Ying et al. 2012). Moreover, the O-linked β-N-acetylglucosamine transferase (OGT) (glycosyltransferase that catalyzes the transfer of GlcNAc from UDP-GlcNAc to serine/threonine residues of target proteins) was shown to be overexpressed in breast cancer cells and in lung, colon and prostate cancer tissue (Caldwell et al. 2010; Itkonen et al. 2013; Mi et al. 2011). Besides, OGT is required for breast cancer growth *in vivo*, for invasion in lung and colon cancer cells and for soft-agar growth in all three cancer types (Caldwell et al. 2010; Mi et al. 2011). In this setting, the HBP through OGT modulates the activity of oncogenic proteins like FoxM1 and c-Myc and cell cycle progression (Caldwell et al. 2010; Itkonen et al. 2013). In cancer cells, HBP also regulates glycolysis, in a process mediated by OGT, HIF and GLUT1. Indeed, the inhibition of O-GlcNAcylation by OGT knock-down leads to an increase in the levels of α-KG, which in turn resulted in HIF-1α degradation (Ferrer et al. 2014).

UDP-glucose metabolism

Glucose-6-phosphate can be deviated to the production of UDP-glucose through the pathway I will refer to as “UDP-glucose pathway”. UDP-glucose is a key intermediate in several processes, namely the formation of glycogen, the production of UDP-galactose, the formation glycosaminoglycans and proteoglycans. UDP-glucose is produced by the enzyme UDP-glucose pyrophosphorylase 2 (UGP2) from glucose-1-phosphate, which in turn, derives from glucose-6-phosphate (see Fig. 17 from Results section, chapter 3.2). UGP2 is the only human enzyme able to catalyze this reaction (Führing et al. 2015).

Glycogen synthesis occurs in the cytosol, initiated by glycogenin, followed by glycogen synthase that elongates the glucose chain by attaching UDP-glucose units through α-1,4 glycocidic linkage. Several types of cancers have shown to present high glycogen content, which can be partially the

result of hypoxia that increases cell storages of glycogen, promoting cancer cell survival (Zois and Harris 2016).

In addition to glycogen, UDP-glucose can generate UDP-glucuronate and UDP-galactose. UDP-galactose supplies galactose moiety for the formation of lactose, glycolipids (cerebrosides), proteoglycans and glycoproteins, while UDP-glucuronate is the donor substrate for glucuronidation reactions, which are important for the deoxification of liophilic hormones and xenobiotics. Additionally, UDP-glucuronate participates in the synthesis of extracellular glycosaminoglycans (such as heparan sulfate and hyaluronan) and of proteoglycans (Kjellén and Lindahl 1991), both of which have important roles in carcinogenesis, for example in the acquisition of a migratory phenotype by breast cancer (Nikitovic et al. 2014). UDP-glucuronate is exclusively synthesized by UDP-glucose dehydrogenase (UGDH) (Sommer, Barycki, and Simpson 2004), an enzyme whose expression is stimulated by androgen and specifically drives excess steroid elimination through glucuronidation, in prostate tumor cell lines (Wei et al. 2009). Clinical and cellular consequences associated with defective UGDH function implicate stable but dynamic association of subunits and suggest that sustaining robust UGDH activity might yield useful therapeutic applications. UDP-glucuronate together with the HBP product UDP-GlcNAc, are the precursors of the hyaluronan (HA) molecule (Weissmann et al. 1954). HA is a large, negatively charged polysaccharide that participates in defining the properties of pericellular matrices - space filling, hydration and provision of a matrix through which cells can migrate - and in transducing signals in proliferating and migrating cells. HA is extruded through the plasma membrane onto the cell surface or into the extracellular matrix while it is being synthesized (Toole 2004), having been extensively implicated in cancer progression (Toole, Biswas, and Gross 1979; Toole 2004).

Mitochondria

Mitochondria are highly dynamic organelles with a crucial role in the maintenance of cellular homeostasis and in regulating cell fate and function. They are considered the “powerhouses of the cell”, as they convert intermediate metabolites, derived from nutrients, into energy in the presence of oxygen - a process termed aerobic respiration. Besides, mitochondria generate metabolites that participate in lipid and nucleotides synthesis. Mitochondria actually house several metabolic pathways such as the TCA cycle, the OXPHOS pathway, fatty acid β-oxidation, parts of the one-carbon metabolism, amino acid metabolism, urea cycle, among others. The number of mitochondria present in each cell can vary from hundreds to thousands, according to the metabolic requirements. Each mitochondrion, in turn, has multiple molecules of its own DNA - the mitochondrial DNA (mtDNA) (Legros et al. 2004; Veltri, Espiritu, and Singh 1990).

Mitochondria are not simply static and isolated organelles, rather forming highly branched, dynamic and motile structures, being the entire mitochondrial population in constant flux, driven by continual fusion and division of mitochondria (Chan 2006).

Mitochondria are signaling organelles, providing cues to the cytosol and initiating biological events under homeostatic and stress conditions (Chandel 2014). Mitochondria regulate programmed cell death, calcium signaling and they are also a major source of ROS, namely superoxide, which originate upon electron leakage from the electron transport chain (ETC). There are different species of ROS and, not only they damage DNA but they can also modify other macromolecules like proteins and lipids. ROS-mediated cell signaling can be pro-tumorigenic until a certain threshold (Weinberg et al. 2010), and when in excess, ROS can be highly damaging to macromolecules (Hamanaka and Chandel 2010).

Mitochondria structure and mtDNA

Mitochondria are bounded by two membranes, the outer and the inner mitochondrial membranes. The outer mitochondrial membrane is more permeable than the inner membrane and contains several proteins and protein-channels like VDAC. The inner mitochondrial membrane area is increased by a large number of infoldings or cristae that protrude into the mitochondrial matrix and maximize its function (Brandt et al. 2017). This membrane contains components of mitochondrial metabolism, such as the complexes of the ETC, the ATP synthase complex, the mitochondrial pyruvate carrier, components of fatty acid oxidation and transport system, and the uncoupling proteins. The matrix is where the mtDNA is localized and where several molecules such as pyruvate and fatty acids are metabolized (Lodish et al. 2013).

The mammalian mtDNA is a maternally inherited circular double-stranded molecule, which has approximately 16,600 base pairs in humans (Falkenberg, Larsson, and Gustafsson 2007). The mtDNA was the first human genome to be sequenced (Anderson et al. 1981) and it encodes 13 peptide-producing proteins, 2 ribosomal RNA and 22 transfer RNA (tRNA) required for their translation (Taanman 1999) (Fig 4). There are several nuclear gene products that play a role in the assembly of the OXPHOS, in the replication of the mtDNA and in the transcription and translation of the mtDNA encoded genes (Kelly and Scarpulla 2004; Scarpulla 2006). Because of its limited repair ability, lack of protective histones proteins and high rate of ROS generation in mitochondria, mtDNA is more susceptible to oxidative damage and has a higher mutation rate compared with nuclear DNA (Lee and Wei 2009). Moreover, considering the fact that mtDNA lacks sizeable introns, mtDNA mutations have a high probability of changing the amino acid composition of the encoded proteins. Thus, many of the mtDNA mutations are likely to have

physiological consequences and might confer a cellular advantage or disadvantage in growth or survival.

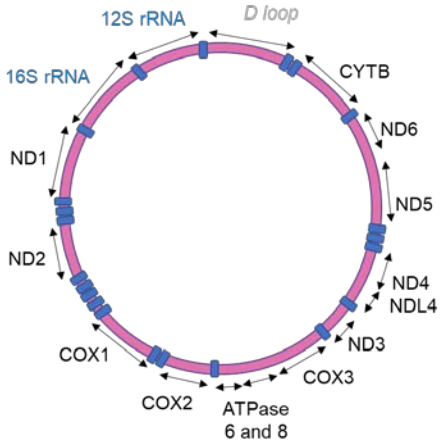

Fig. 4: Human mtDNA. It contains 13 peptide-producing proteins, 2 rRNA (names in blue) and 22 tRNAs (represented by blue rectangles). D-loop is a non-coding region. rRNA, ribosomal RNA; tTNA, transfer RNA COX, cytochrome c oxidase; CYTB, cytochrome b; ND, NADH dehydrogenase.

Mitochondrial metabolism

Among the several metabolic reactions taking place in the mitochondria, the TCA cycle and the OXPHOS will be described in more detail, since they are object of study of this thesis.

In the TCA cycle (also called the Krebs cycle), carbon fuels like carbohydrates, fatty acids and amino acids are oxidized, most of them entering the cycle as acetyl-coenzyme A (acetyl-CoA). The TCA cycle can also produce precursors for the generation of lipids, proteins modifications or signaling molecules. Furthermore, some reactions can replenish components of the TCA cycle, a process known as anaplerosis. In the OXPHOS, high energy electrons are donated to the ETC to generate a proton gradient across the membrane that culminates in the generation of ATP and concomitant transfer of electrons to oxygen.

TCA cycle and OXPHOS

Pyruvate is the end product of glycolysis and occupies a key node in the regulation of carbon metabolism, since it is at the intersection of several catabolic and anabolic pathways. Pyruvate

can be utilized for mitochondrial acetyl-CoA generation, which is then subjected to oxidation in the TCA cycle.

Import of pyruvate across the inner mitochondrial membrane requires the mitochondrial pyruvate carrier (Bricker et al. 2012; Herzig et al. 2012), after which it is oxidized to acetyl-CoA by the PDH complex. Acetyl-CoA is subsequently converted to citrate via condensation with oxaloacetate (OAA) by citrate synthase. In turn, citrate is converted, by aconitase, to isocitrate, which in turns generates α-KG through IDH. This enzyme is one of the main points of regulation of the TCA activity and during this reaction NAD^+ or $NADP^+$ is reduced to NADH or NADPH, respectively. α-KG dehydrogenase generates succinyl-CoA from the oxidation of α-KG, reducing NAD^+ to NADH. The reaction of succinyl CoA synthetase, the formation of succinate from succinyl CoA, is linked to a substrate-level phosphorylation of GDP to GTP, which is energetically equivalent to the formation of ATP. The next reaction is catalyzed by SDH, which is an enzyme that not only belongs to the TCA cycle but also in inserted in the ETC, constituting complex II. In the TCA cycle, it catalyzes the oxidation of succinate to fumarate, concomitant with the reduction of FAD to $FADH_2$. Then, water is added to fumarate, converting it to malate. In the last step of the TCA cycle, OAA is regenerated by oxidation of malate. Another molecule of NAD^+ or $NADP^+$ is reduced to NADH or NADPH in the process (Fig. 5).

In some conditions, the uptake of pyruvate by the mitochondria can be bypassed by several alternative pathways that feed the TCA cycle and the OXPHOS-mediated generation of ATP. These include the malic enzyme, the pyruvate-alanine cycle, glutamine or fatty acids (Bender and Martinou 2016).

Electrons obtained from the oxidative reactions of the TCA cycle are shuttled through NAD^+/NADH and FAD/$FADH_2$ to the ETC, creating an electrochemical gradient that fuels ATP production.

The multimeric protein complexes that constitute the ETC carry out redox reactions, where electrons are transferred from the reductive equivalents (NADH and FADH2) to the electron final acceptor (oxygen), in a process named oxidative phosphorylation (OXPHOS) (Hatefi 1985). The ETC is composed of 77 nuclear encoded and 13 mtDNA encoded subunits that assemble and function as a large complex. These subunits are organized into four complexes: complex I or NADH:ubiquinone oxiredutase, complex II or SDH, complex III or ubiquinol-cytochrome c oxiredutase and complex IV or cytochrome c oxidase (COX). These complexes are formed by flavoproteins, ubiquinone (or coenzyme Q), cytochromes, iron-sulfur proteins and cupric protein, being cytochrome c and ubiquinone two mobile components.

Complexes I and II accept electrons from NADH from $FADH_2$, respectively, and both complexes transfer them to the ubiquinone pool. This molecule is thus reduced, being named ubiquinol.

Ubiquinol, a motile and hydrophobic molecule donates the electrons to complex III. Electrons then move to cytochrome c and are transferred to complex IV, where they reduce molecular oxygen to water. As the electrons flow in the ETC, the released energy is used by complexes I, III and IV to pump protons across the inner mitochondrial membrane to the intermembrane space. The ETC generates, therefore, a proton concentration gradient and an electric potential (voltage gradient), collectively called proton-motive force. This chemiosmotic coupling is responsible for driving the energy-requiring process, ATP synthesis. ATP synthase complex (sometimes referred to as complex V) is a member of ATP-powered proton pumps. Protons flow back to the matrix through ATP synthase promoting ATP synthesis by the enzyme (Fig 5). Matrix ATP is then exchanged for cytosolic ADP by the inner membrane ADP/ATP translocase (Lodish et al. 2013).

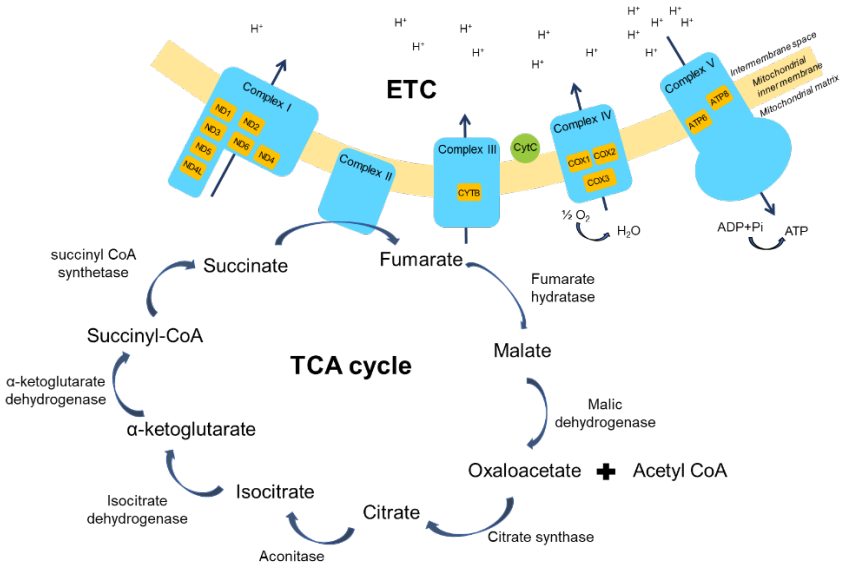

Fig. 5: TCA cycle and ETC (simplified versions). The mtDNA-encoded proteins that constitute the complexes are depicted. NADH dehydrogenase, ND; COX, cytochrome c oxidase; O_2, oxygen; H_2O, water; CytC, cytochrome c; H^+, protons.

The OXPHOS produces up to 38 molecules of ATP as energy currency, while glycolysis yields 2 ATP per molecule of glucose, being, therefore, OXPHOS considered much more efficient.

Owing its central role in metabolism, defects in the mitochondrial respiratory chain underlie a spectrum of human conditions, mostly related with degenerative disease and affecting primarily high energy-demanding tissues such as skeletal muscle, cardiac muscle and brain. Besides degenerative conditions, one must ask:

What about mitochondrial metabolism in cancer?

Warburg speculated that the aerobic glycolysis phenotype of cancer cells was triggered by a OXPHOS-impairing mitochondrial defect (Warburg 1956); however, Warburg himself failed to demonstrate defective respiration as a general feature of malignant cells (Koppenol, Bounds, and Dang 2011). In fact, many studies have analyzed mitochondrial function of cancer cells and showed that mitochondrial defects do not account for the phenomenon in general (Crabtree 1929). Indeed, respiration and other mitochondrial activities are often required for tumor growth. For example, intermediates from the TCA cycle are essential to complement other metabolites from glycolysis, supporting diverse pathways required for proliferation (Ahn and Metallo 2015). Proliferating cancer cells appear to reprogram their metabolism towards favoring glycolysis in a regulated way, instead of an adaptation to a OXPHOS dysfunction, and the majority of cancers are able to revert back to OXPHOS when lactic acid generation is inhibited (Fantin, St-Pierre, and Leder 2006). A study using a tetracycline-inducible *KRas*-driven PDAC mouse model showed that, upon oncogene ablation, the surviving cells, responsible for tumor relapse and with cancer stem cell features, have more active mitochondria, a strong reliance on mitochondrial respiration and impaired glycolysis (Viale et al. 2014). Birsoy *et al.* used a continuous-flow culture system to expose several cell lines to low glucose media and showed that OXPHOS is the key metabolic process required for optimal proliferation of cancer cells under glucose limitation. The low-glucose sensitivity was associated with deficiencies in glucose utilization or mtDNA mutations in complex I genes (Birsoy et al. 2014). Besides, cancers are heterogenous and dynamic entities, where different types of metabolism coexist (Hensley et al. 2016; Viale et al. 2014).

Nevertheless, certain malignancies indeed harbor mitochondrial defects that make aerobic glycolysis a crucial adaptation. Mitochondrial dysfunction can be due to genetic alterations, either in mtDNA or in nuclear DNA encoded mitochondrial proteins, or to biochemical defects of mitochondria caused by deregulated pathways and oncogenic activation or inactivation of tumor suppressor genes. The presence of mtDNA mutations has been recognized in a wide variety of human cancers (Copeland et al. 2002; Fliss et al. 2000; He et al. 2010; Petros et al. 2005). An interesting study stressed the role of mtDNA mutations in tumor progression. By exchanging the mtDNA of two mouse cell lines with different metastatic potentials, the capability to metastasize *in vivo* accompanied the mtDNA (Ishikawa et al. 2008). It was also demonstrated that the metastatic cell lines harbored a defective mitochondrial complex I, caused by a mtDNA mutation. Such mtDNA mutation triggered ROS overproduction which, in turn, upregulate nuclear genes such as *HIF-1α* and *VEGF*, involved in neoangiogenesis and, consequently, regulate tumor cell metastasis (Ishikawa et al. 2008).

The analysis of mtDNA mutations and their relevance for tumorigenesis is usually performed using cybrid cell lines. Cybrids are cytoplasmatic trans-mitochondrial hybrids, which are created by repopulating a cell line depleted of mtDNA, termed ρ0 (rho 0), with exogenous human mitochondria, as it was first demonstrated by King and Attardi (King and Attardi 1989). Since then, the use of cybrid technology has been growing exponentially. Indeed, ρ0 cells can be fused with an enucleated cell line or even platelets that carry the desirable mtDNA mutations. Importantly, this technology allows the differentiation of the effects caused by mtDNA mutations from those caused by nuclear DNA, since the cybrids cell lines have the same nuclear background (Swerdlow 2007).

Mitochondrial defects can also arise from genetic alterations in the genes that encode TCA cycle proteins. These will be described below in the next section.

Mitochondrial metabolism is also important to feed other anabolic pathways. For example, aspartate (that can be produced from the TCA intermediate OAA) has been shown to be one of the key players in cancer cell proliferation and dependent on the presence of an electron acceptor by two back-to-back studies (Birsoy et al. 2015; Sullivan et al. 2015). Indeed, respiration has an important role in sustaining cancer cell proliferation because it produces electron acceptors for aspartate synthesis (Birsoy et al. 2015; Sullivan et al. 2015). Upon OXPHOS inhibition, cells become dependent on the production of aspartate from OAA through the aspartate transaminase GOT1 in the cytosol (Birsoy et al. 2015). In PDAC, oncogenic *KRas* redirects glutamine metabolism towards the production of aspartate, which is further metabolized in the cytosol into OAA by GOT1, and finally generating malate and pyruvate, in order to maintain a high $NADPH/NADP^+$ ratio and the cellular redox state (Son et al. 2013).

Metabolic enzymes as tumor suppressors or oncogenes

The first example of causality between mitochondrial dysfunction and tumorigenesis was the observation that the genes encoding for *SDH* and *FH* predispose to hereditary neoplasias (Gottlieb and Tomlinson 2005). In fact, the identification of mutations in genes that encode mitochondrial metabolic enzymes was key to reinforce the role of metabolism in tumorigenesis.

SDH, or complex II of the respiratory chain, is an heterotetrameric enzyme and the only ETC complex whose subunits are all encoded by the nuclear DNA. This complex, which establishes a direct link between the TCA cycle and the OXPHOS, in mammals is classically formed by four structural subunits - SDHA, SDHB, SDHC, SDHD and several prosthetic groups – and assembled by several proteins such as SDHAF1 and SDHAF2 (Rutter, Winge, and Schiffman 2010). SDHA is the catalytic unit responsible for oxidation of succinate to fumarate, collecting the resulting

electrons and reducing FAD. Then the electrons are transferred to the iron-sulphur centers in SDHB (iron-sulphur protein). Finally, the electrons are moved to the cytochrome b and ubiquinone-associated components SDHC and SDHD, which anchor this complex to the mitochondrial inner membrane.

Germline mutation in the genes encoding SDH subunits are predominantly linked to the development of inherited phaeochromocytoma and/or paraganglioma. Loss of function mutations have been characterized in *SDHA*, *SDHB*, *SDHC* and *SDHD* genes, as well as in the assembly factor *SDHAF2* (Astuti, Douglas, et al. 2001; Astuti, Latif, et al. 2001; Baysal et al. 2000; Burnichon et al. 2010; Gimm et al. 2000; Hao et al. 2009; Niemann and Müller 2000).

Paragangliomas and phaeochromocytomas are rare tumors that arise in the autonomous nervous system, specifically from extra-adrenal thoracic and abdominal paraganglia and from the sympathetic lineage-derived cells from the adrenal medulla. The distinction between paraganglioma and phaeochromocytoma is based on their site of origin: phaeochromocytomas develop in the medulla of the adrenal gland, while the term paragangliomas is used for tumors that arise in extra-adrenal paraganglia, occurring in the abdominal region or in the head and neck region, a distinction that also reflects differences in tumor behavior (Dahia 2014).

We identified a founder *SDHB* mutation in Portuguese paraganglioma and pheochromocytoma patients (Martins and Nunes et al. 2013 - manuscript in annex I). Our patient cohort of these neuroendocrine neoplasias showed a high prevalence of germline *SDH* mutations (20 out of 37 patients; 54.1%), a finding that is most likely due to the prevalence of a single deletion in *SDHB* that encompasses the promotor region plus exon 1. This deletion accounted for 55% of all patients with *SDH* mutations. The observation that the deleted allele shows the same haplotype in all patients suggested a founder effect for this particular deletion, being the first *SDHB* founder mutation described in the Portuguese population (Martins and Nunes et al. 2013 - manuscript in annex I).

In addition to mutations in hereditary neoplasias, SDH is also found downregulated in sporadic cancer such as HCC, where it is associated with advanced tumor stage and poor survival (Tseng et al. 2018). Decreased SDHB in HCC cells also leads to bioenergetic and an increase in glycolytic enzymes (Tseng et al. 2018).

The enzymatic reaction subsequent to SDH is catalyzed by FH that converts fumarate into malate. Germline heterozygous mutations in *FH* were found to cause hereditary leiomyomatosis and renal cell cancer syndrome, a hereditary cancer syndrome characterized by the presence of benign tumors of the skin and uterus, and a highly malignant form of renal cell cancer (The Multiple Leiomyoma Consortium 2002).

The mechanism of tumorigenesis in these cancer syndromes is still matter of debate. It has been suggested that loss-of-function mutations lead to an accumulation of succinate and fumarate, inhibit prolyl hydroxylases and consequently result in HIF-1α stabilization and HIF-1 activation (Isaacs et al. 2005; Pollard et al. 2005; Selak et al. 2005). A ROS-dependent mechanism for HIF-1α stabilization has also been proposed for *SHDB* mutations (Guzy et al. 2008). In the case of *FH* mutations, fumarate accumulation can dysregulate the metabolism of cells. For example, it has been shown that fumarate can cause the succination of proteins - a non-enzymatic covalent modification of reactive cysteine residues - like aconitase 2 or iron-sulfur cluster biogenesis family of proteins, impairing their enzymatic activity, which results in altered of TCA cycle function, complex II inhibition and decreased respiration (Frezza et al. 2011; Ternette et al. 2013; Tyrakis et al. 2017). Interestingly, it has been shown that concomitant loss of complex I activity is necessary for the metabolic phenotype of SDH mutant tumors (Lorendeau et al. 2017). Additionally, both SDH and FH loss have been associated with epithelial to mesenchymal transition (EMT), which confers migratory and invasive properties to cancer cells, which is described in more detail in the discussion of this thesis (Aspuria et al. 2014; Sciacovelli et al. 2016; Tseng et al. 2018; Wang, Chen, and Wu 2016).

Another striking example that links mitochondrial dysfunction with tumorigenesis was the groundbreaking discovery of *IDH* mutations, first in brain tumors, and then in other cancer types (Balss et al. 2008; Borger et al. 2012; Kosmider et al. 2010; Mardis et al. 2009; Watanabe et al. 2009; Yan et al. 2009). Mutations in *IDH1* and *IDH2*, which encode the (predominantly) cytosolic and mitochondrial forms of $NADP^+$-dependent IDH, respectively, were shown to be very frequent events in malignant gliomas, in particular astrocytomas and oligodendrogliomas (Yan et al. 2009). Interestingly, these *IDH1* and *IDH2* mutations give rise to neomorphic enzymes that produce D-2-HG instead of α-KG (Dang et al. 2009). This oncometabolite can function as a competitive inhibitor of α-KG-dependent dioxygenases, including histone demethylases and the 5-methylcytosine dioxygenases TET (ten-eleven translocation) enzymes (Figueroa et al. 2010; Xu et al. 2011), having an important impact on DNA epigenetic and protein posttranslational modifications. 2-HG is a chiral molecule and has an L- enantiomer produced by LDHA or malate dehydrogenase, which besides being a potent inhibitor of α-KG-dependent enzymes, also leads to the stabilization of HIF (Intlekofer et al. 2017; Xu et al. 2011). L-2-HG is associated with brain and renal cancers (Haliloglu et al. 2008; Shim et al. 2014).

The role of succinate, fumarate or 2-HG as oncometabolites illustrates how metabolic enzymes can promote transformation through non-metabolic effects.

Glutamine

Glutamine metabolism in cancer cells has been the subject of a lot of research in the last years. Glutamine metabolism provides both anaplerotic and NADPH demands of growth, allowing cells to use glucose carbon to build nucleic acids and lipids, while contributing for protein synthesis.

Glutamine metabolism can be used to support the nitrogen demand imposed by nucleotide synthesis, or for the maintenance of nonessential amino acid pools and nitrogenous biomolecules (Meng et al. 2010; Zetterberg and Engström 1981). Glutamine can be converted directly into glutamate by GLS, which in turn can produce α-KG by several enzymes such as glutamate dehydrogenase (GDH) or transaminases. Mammary proliferating cells shift from a nitrogen-wasting mode, where α-KG is preferentially produced from glutamate by GDH, to a mode where this reaction is catalyzed by transaminases in a nitrogen-sparing mode, in order to synthesize nonessential amino acid (Coloff et al. 2016).

Tumor cells consume large amounts of glutamine, which can provide both carbon and nitrogen (Sauer, Stayman, and Dauchy 1982). For instance, glioblastoma cells show a high rate of glutamine metabolism that is used, not only for protein and nucleotide biosynthesis, but also to produce NADPH to fuel lipid synthesis (and nucleotide biosynthesis) (DeBerardinis et al. 2007). Pancreatic tumors harboring an oncogenic *KRas* are dependent on glutamine metabolism and specifically on the cytosolic conversion of glutamine-derived aspartate into OAA, malate and then pyruvate. This is important to increase the NADPH/$NADP^+$ ratio and maintain the redox control (Son et al. 2013). However, the use of GLS inhibitors has shown no antitumor properties in an *in vivo* model of PDAC, indicating that cancer cells can rewire their metabolism in order to utilize the available nutrients to sustain proliferation (Biancur et al. 2017). Moreover, glutamate is highly secreted by triple-negative breast cancer cells and promotes HIF-1α stabilization in a paracrine way. This happens because extracellular glutamate inhibits cystine uptake through the xCT glutamate-cystine antiporter, causing intracellular cysteine depletion which directly inhibits the HIF-α prolyl-hydrolase, EglN1 (Briggs et al. 2016). Glutamate is also a precursor of glutathione, a major cellular antioxidant (Welbourne 1979). Glutathione is one of several glutamine metabolism products that directly control ROS levels. Glutathione levels play an important role in tumorigenesis and drug resistance (Godwin et al. 1992).

The high glutamine consumption *in vitro* may, however, be a consequence of the culture media conditions. NSCLC do not seem to rely on glutamine for growth since GLS inhibitors are not very effective in slowing tumor growth; furthermore, when nutrient tracing was performed *in vivo*, glucose, rather than glutamine, contributes more for the formation of TCA cycle intermediates in NSCLC tumors (Davidson et al. 2016). The same resistance to GLS inhibitors has also been described in a mouse model of PDAC (Biancur et al. 2017). In fact, when NSCLC

cells were cultured in adult bovine serum, whose composition is more similar to human plasma than the standard media, they consumed less glutamine and responded to GLS inhibitors in a similarly way to how on-site tumors. After deconvoluting the differences in the cell culture conditions, cystine seems to account for the differential glutamine anaplerosis and dependence of these different environments (Muir et al. 2017).

Research on glutamine highlighted the fact that nutrients other than glucose play an important role in cancer cells and prompted interest in other metabolites like serine and glycine, branched chain amino acids, lactate, acetate and other fatty acids as cancer fuels.

Metabolism of tumors: much more than cancer cells

Cancer cell metabolism can be modulated by the microenvironment, namely through the availability of nutrients and the presence of other cell types. Tumors, like an organ, are complex ecosystems, being composed of much more than solely cancer cells. They integrate several different cell types such as immune cells, endothelial cells, fibroblasts, normal epithelial cells, among others (Egeblad, Nakasone, and Werb 2010). Cells that constitute the tumor stroma compete with tumor cells for nutrients, oxygen and space, while secreting growth factors, cytokines, ECM components and metabolites, in this way promoting tumor heterogeneity and shaping the metabolic requirements of cancer cells.

A metabolic symbiosis between the stroma and cancer cells has in fact been described. It has been proposed that stromal cells exhibit aerobic glycolysis and promote tumor growth by the secretion of energy-rich metabolites, such as pyruvate and lactate, which are used by tumor cells. This phenomenon has been named “the reverse Warburg effect” (Pavlides et al. 2009).

The metabolism of cancer cells can also regulate a phenomenon called “epithelial defence against cancer” (EDAC) that characterizes the initial stages of carcinogenesis and refers to the competition between normal cells and the transformed epithelial cells for survival and space, which results in the non-immune elimination of cancer cells from epithelial tissues. Ras-transformed cells, where EDAC has been shown, present increased glycolysis and reduced mitochondrial function, characterized by PDK4 upregulation, a metabolism that is potentiated by the neighboring normal epithelial cells and it is determinant for their elimination by apical extrusion (Kon et al. 2017).

The metabolism of tumor cells can also alter stromal cells’ function. For example, lactate produced by cancer cells can modulate the immune response, since it enhances the production of proinflammatory cytokines by monocytes and macrophages stimulated with Toll-like receptor ligands (Shime et al. 2008). Besides, the microenvironment acidification, resulting from the lactic

acid secreted by tumor cells, leads to an upregulation of arginase 1 in tumor-associated macrophages, which is characteristic of the M2 phenotype (Ohashi et al. 2013). The metabolism of cancer cells can also contribute for immune escape. Indeed, tumor extracellular acidity is associated with an impaired effector function of $CD8^+$ tumor specific T lymphocytes in human and mouse (Calcinotto et al. 2012). Moreover, glucose consumption by tumors metabolically restricts $CD8^+$ T cells, leading to their dampened mTOR activity, glycolytic capacity, and interferon-γ production, thereby allowing tumor progression (Chang et al. 2015). Phosphoenolpyruvate, a glycolytic intermediate, is important for $CD4^+$ T cells anti-tumor activity, because it sustains calcium-NFAT signaling and T cell activation by repressing sarco/ER Ca^{2+}-ATPase-mediated calcium re-uptake to the endoplasmic reticulum (Ho et al. 2015).

Not only immune cells, but also fibroblasts are affected by lactate. It has been shown increased levels of lactate stimulate hyaluronic acid production by fibroblasts, which may contribute for increased tumor cell invasiveness (Stern et al. 2002). Lactate promotes angiogenesis by stabilizing NDRG3 protein, which binds c-Raf to mediate hypoxia-induced activation of Raf-ERK pathway in tumor cells (Lee et al. 2015). Besides, lactate-induced signaling in endothelial cells may stabilize HIF-1α and activate an autocrine NFB/interleukine-8 pathway (Sonveaux et al. 2012; Végran et al. 2011).

Summing up, since the first experiments made by Otto Warburg, research on tumor metabolism has experienced a huge rise, to the point of being considered a hot-topic in cancer research. In accordance with the fact that cellular metabolism is dynamically modulated and adjusted in response to various conditions, tumors show metabolic adaptations that result from a combination of the tissue of origin, the genetic alterations they acquire and their surroundings. Although we know better how genetic alterations and stromal cells can tailor cellular metabolism, exposing metabolic vulnerabilities, much less is known about the influence of the cell lineage.

1.2 - CELL MIGRATION

1.2.1 - How do cells migrate?

Cells can adopt different strategies to migrate, engaging on various signaling programs, depending on the ECM nature. Tumor cells, for example, can present several types of cell movement, from individual to collective and from amoeboid to mesenchymal migration (Friedl and Alexander 2011). Migration and invasion (the latter also referred to as invasive or mesenchymal migration) are two denominations commonly used to distinguish the coordinated cell adhesion and contractility of cells from the movement that also involves proteolytic remodeling of the ECM. Amoeboid movement, which is less dependent on proteases, is characterized by the loss of cell polarity, detachment from the ECM - leading to a rounded morphology - and movement through the path of least resistance and has been reported *in vivo* in leukocytes and also in tumors (especially liquid tumors) (Friedl and Wolf 2003). On the other hand, migration that implies matrix-degrading enzymes is particularly relevant in the context of cancer metastasis.

Invasive migration of single cells is intimately related with cell adhesion and cell polarity and implies a change in cells' shape and stiffness, in order to interact with the surrounding tissue structures. This process encompasses the formation a leading edge protrusion mediated by the polymerization of actin; adhesion to ECM which is generally mediated by integrins; contact-dependent ECM cleavage by physical force or proteases; cytoskeleton contraction (mediated by actomyosin) of the cell body increasing longitudinal tension; and finally, trailing edge detachment and retraction, with final translocation of the cell body (Friedl and Wolf 2003) (Fig 6).

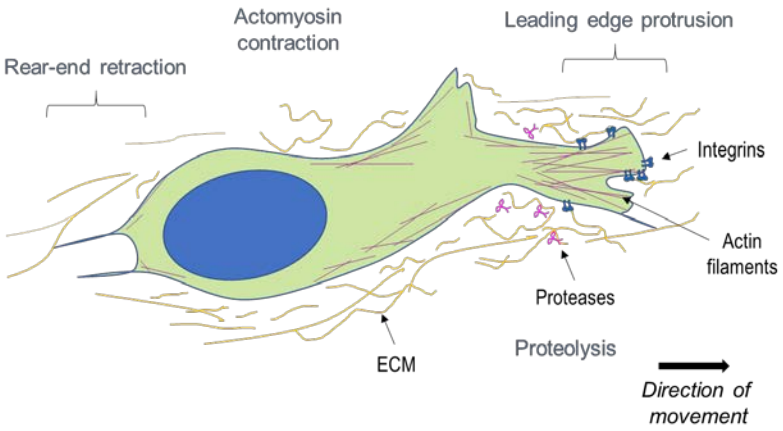

Fig. 6: Invasive migration mechanism by the formation of pseudopod protrusions. ECM, extracellular matrix.

Mechanistically, invasive migration requires the interaction with the ECM through integrins (in most situations) that become locally enriched, cluster and stabilize forming a focal contact. Besides, invasive migration entails a dynamic cascade of cytoskeleton rearrangements coordinated by Rho family GTPases. These key effectors of migration will be now described in more detail.

1.2.2 - Key effectors in migration

Integrins

Integrins are key elements for invasive migration, as they generate the adhesive interaction and traction toward the ECM substrate (Palecek et al. 1997). The importance of integrin-β1 for cell migration and for *in vitro* invasiveness was first highlighted in studies using anti-integrin antibodies, where it was shown that they can block cell migration and invasion (Yamada et al. 1990). In breast cancer cells, integrin-β1 was shown to be required for 3D matrigel invasion and for the contraction of the rear pole of the invading cell (Poincloux et al. 2011). Integrins are transmembrane proteins that bind extracellularly to the ECM components, such as fibronectin, laminin, vitronectin, collagen and various other adhesive glycoproteins (Humphries, Byron, and Humphries 2006). They form heterodimers composed of α and β chains, and the existence of several of α and β subunits generates numerous combinations with different properties (Humphries, Byron, and Humphries 2006). For example, integrin-β1 dimerizes with several α subunits, including integrin-α5 and, in that case, mediates fibronectin-dependent migration, constituting a prototypic integrin-ligand pair (Takagi et al. 2003). Upon binding to the ECM, integrin dimers form clusters in the cell membrane and, through their intracellular domains bind several cytoskeletal-associated and adaptor proteins, including talin, paxillin, kindlin, vinculin and BCAR1 (breast cancer anti-estrogen resistance 1; also called p130Cas) that ultimately control cytoskeletal organization and intracellular signaling (Geiger, Spatz, and Bershadsky 2009). Integrins can sometimes be classified according to the structural basis of the molecular interaction between them and the ligand partner. For example, several members of the integrin family, such as integrins β1α5 and β1α8, recognize an adhesive epitope composed by the three peptides arginine-glycine-aspartic acid (RGD) in ECM molecules (Humphries, Byron, and Humphries 2006). Furthermore, integrins are highly regulated molecules. Integrins are glycoproteins and it has been shown that in the case of integrin-β1, N-glycosylation is determinant for its function (Isaji et al. 2009). Finally, integrins not only regulate intracellular signaling pathways that control cytoskeletal organization, force generation but also cell proliferation and survival (Lane et al. 2010; Moreno-Layseca and Streuli 2014; Sethi et al. 1999).

As previously mentioned, integrins transduce signals from the extracellular environment, through the association of their intracellular domain with adaptor proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors. The dynamic intracellular structures formed by the links between integrins and the actin cytoskeleton, which transmit and respond to mechanical forces, are denominated focal adhesions (Bershadsky, Balaban, and Geiger 2003). Focal adhesion kinase (FAK) and Src are two protein kinases commonly activated by integrin signaling and regulate migration by phosphorylation events. FAK can have a kinase dependent or kinase independent function. The kinase dependent functions of FAK have a major role in migration, particularly the autophosphorylation site on tyrosine (Y) 397, which occurs after clustering, was shown to be specifically relevant to increase migration (Cary, Chang, and Guan 1996; Ritt, Guan, and Sivaramakrishnan 2013; Toutant et al. 2002). Clustering in the lipid biolayer and consequent activation of FAK was shown to be induced by phosphatidylinositol 4,5-bisphosphate, which promotes efficient autophosphorylation of Y397 and likely enhances integrin clustering as well as the scaffolding function of FAK (Goñi et al. 2014). Besides, growth factor receptor signaling such as platelet-derived growth factor (PDGF) receptor, epidermal growth factor (EGF) receptor, hepatocyte growth factor (HGF)/Met and RET kinase can also trigger FAK activation (Chen and Chen 2006; Plaza-Menacho et al. 2011; Sieg et al. 2000). The phosphorylation of residue Y397 of FAK creates a docking site for proteins with Src homology 2 domains, including Src family kinases and PI3K (Cary, Chang, and Guan 1996; Chen et al. 1996; Cobb et al. 1994). After binding to FAK, Src phosphorylates several tyrosines, such as the ones located in the activation loop (Y576 and Y577) which confer full catalytic activity (Calalb, Polte, and Hanks 1995). The activity of Src is indeed required for integrin-regulated spreading and migration (Cary et al. 2002). The activated FAK/Src complex has been implicated in the tyrosine phosphorylation of several focal adhesion proteins, such as paxillin (Schaller and Parsons 1995) and p130Cas (Tachibana et al. 1997).

Rho GTPases

Downstream effectors of integrins and FAK include the small Rho family GTPases, which reinforce cell protrusion and rear contraction. This family of small signaling G proteins, which also regulates cell adhesion and polarity, includes several subfamilies being RhoA, Rac-1 and Cdc42 the most studied members. Rho, via the Rho kinase promotes the formation of cytoplasmic stress fibers, stabilization of focal contacts into mature focal adhesions and cell body contraction through activation of the actomyosin cytoskeleton (Amano et al. 1997; Kimura et al. 1996), being associated with rounded/amoeboid form of movement (Sahai and Marshall 2003). On the other hand, Rac1 is regulated by DOCK3 (dedicator of cytokinesis 3), in association with the adaptor

molecule NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) and, together with WASF2 (WAS protein family member 2), drives mesenchymal movement and suppress amoeboid movement (Sanz-Moreno et al. 2008). Rac-1 and Cdc42 are considered the main regulators of membrane protrusions into lamellipodia and filipodia (Aspenström 1999), although this may be context-dependent (Borm et al. 2005). Rho GTPases can be activated in response to growth factors and respective receptors (Nobes et al. 1995; Ridley and Hall 1992).

Matrix metalloproteinases (MMPs)

One of the key steps in invasive migration is the ECM degradation that modifies the molecular and mechanical tissue properties, allowing space for the advancing cells or groups of cells (Friedl et al. 1997; Wolf et al. 2007). For this to happen, cell surface proteases become engaged with extracellular scaffold proteins that compose the ECM and execute locally controlled proteolysis (Friedl and Wolf 2003). The proteolysis is catalyzed by several proteases such as matrix metalloproteinases (MMPs), serine and cysteine proteases and ADAMs (a disintegrin and metalloproteases). MMPs are zinc-dependent endopeptidases. They are synthetized as inactive zymogens, activated by proteinase cleavage and can either be secreted or stay covalently linked to the cell membrane. MMPs cleave not only ECM structural proteins, but also other proteinases, growth-factor-binding proteins, growth-factors, receptor tyrosine kinases, as well as cell-adhesion molecules, such as E-cadherin, integrins or CD44. Their activity can be regulated by endogenous inhibitors such as α2-macroglobulin or thrombospondin-2 (Egeblad and Werb 2002). Therefore, MMPs also promote invasion by activating signaling pathways involved in invasion. Moreover, ECM degradation generates biologically active epitopes of ECM components with adhesion- or migration-promoting effects (Giannelli et al. 1997; Kenny et al. 2008). Together, the degradation of the ECM creates a pro-invasive microenvironment, that is particularly relevant for cancer cell invasion.

1.2.3 - Cancer metastasis

As previously mentioned, there are several types of cell movements implicated in cancer invasion and metastasis. Metastasis in epithelial tumors is a complex and multistep process (Fig. 1) that is not fully understood. However, cell migration is widely recognized to be an important prerequisite for cells to be able leave the primary tumor and colonize secondary tumor sites (Sträuli and Weiss 1977). One of the cellular programs that enables cells to acquire migratory features is EMT and it will be discussed in chapter 1.3.

The molecules that mediate migration are commonly dysregulated during cancer. MMPs are overexpressed in many human cancers, having been considered hallmarks of cancer due to their "path-clearing" role and their signaling properties (Egeblad and Werb 2002). For example, several MMPs are overexpressed in breast malignant tumors as compared with normal tissue (Garbett, Reed, and Brown 1999) and some of them are actually expressed by components of the tumor microenvironment (Iwata et al. 1996). It has also been shown in several tissue types that MMPs promote carcinogenesis (Egeblad and Werb 2002). The deficiency of MMP-9 in a transgenic mouse model of skin cancer results in reduced keratinocyte hyperproliferation at all neoplastic stages and a decreased incidence of invasive tumors. Interestingly, the tumors that arise in the absence of MMP-9 are of a more advanced stage than those that arise in wild-type mice. Moreover, the restoration of MMP-9 expression by bone-marrow transplantation of MMP-9-expressing cells leads to a restoration of skin carcinogenesis (Coussens et al. 2000).

Not only MMPs but many of the previously described migration effectors are commonly altered in cancer and play an important role in carcinogenesis. Cancer progression is frequently characterized by changes in the composition and organization of ECM. Fibronectin, for example, promotes epithelial cell growth during mammary gland tumorigenesis (Williams et al. 2008) and, in ovarian cancer, it is secreted by mesothelial cells mediating cancer cell invasion (Kenny et al. 2014). The molecules that transduce the signals conveyed by cell adhesion to the ECM also influence tumorigenesis and progression. Integrin-αvβ3 is more expressed in angiogenic blood vessels compared to quiescent preexisting blood vessels and it is required for their infiltration into the tumor mass (Brooks, Clark, and Cheresh 1994). The same integrin was also shown to be required for prostate cancer bone metastasis by promoting the adhesion and migration of cancer cells to the bone microenvironment and subsequent tumor growth (McCabe et al. 2007). Integrin-α5β1 mediates breast cancer cell migration and invasion induced by angiopoietin-2 and the downstream signaling cascade involving Akt, glycogen synthase kinase (GSK)-3β, Snail, and E-cadherin (Imanishi et al. 2007). During the process of metastasis formation in the lungs, integrin-β1 and FAK play an important role in adhesion-related signaling events and proliferation after extravasation. The expression of integrin-β1 induces the activation of FAK that regulates the proliferation of cancer cells scattered within the ECM (Shibue and Weinberg 2009). Preclinical and clinical trials of small molecule FAK inhibitors that affect both tumor cell and stroma, have demonstrated that FAK can be an effective cancer target in various cancers (Yoon and Dehart et al. 2015). Interestingly, FAK seems to have a role in modulating cellular metabolism glucose, lipid and glutamine metabolism that contributes for tumor cell rapid growth, survival and invasion (Zhang and Hochwald 2014).

1.3 – EPITHELIAL TO MESENCHYMAL TRANSITION

EMT is the reversible process by which epithelial cells acquire mesenchymal properties, such as increased migration and invasion, that are leveraged by alterations in adhesion and cytoskeleton molecules and in microenvironment modulators. EMT is sometimes accompanied by changes in cell proliferation and survival.

EMT is characterized by a gene expression landscape that reflects the phenotypic/morphological changes that cells undergo, depending on the cellular context. Some transcription factors, namely Snail, Slug, zinc finger E-box binding homeobox (Zeb) 1 and 2 and Twist, have a particularly prominent role as master coordinators of the gene expression changes that occur during EMT. They are induced upon the activation of several signaling pathways that occur in response to extracellular cues, such as the cytokines transforming growth factor (TGF)-β or tumor necrosis factor (TNF)-α, the growth factors HGF, EGF, or even nutrient and oxygen availability.

EMT is an essential process in embryonic development and tissue repair and also contributes to progression of disease namely fibrosis and cancer. Each of these types of EMT will be explored in subsequent sections.

1.3.1 - EMT phenotype

The epithelial phenotype reflects the organization of a population of cells. Epithelial cells have three main features: (i) the cells are closely opposed to one another and, at the interface, present cell-to-cell adhesion molecules that form cell junctions; (ii) they exhibit functional and morphological apico-basal polarity and (iii) their basal surface is attached to a basement membrane (although in epithelioid tissues, cells lack the apical domain) (Pawlina and Ross 2015) (Fig 7).

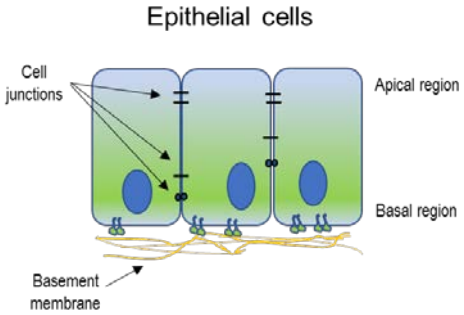

Fig. 7: Epithelial cell morphology, which is characterized by multiple cell-cell contacts, apico-basal polarity and the presence of a basement membrane.

During EMT there is suppression of molecules that define the epithelial cell state and, at the same time, cells acquire mesenchymal properties. EMT was first described in the context of embryonic development, as a mechanism for tissue remodeling and a key event to diversify the structure of organisms. The mesenchyme is an embryonic precursor tissue with three dimensions, that generates a range of structures in vertebrates including cartilage, bone, muscle, kidney, and the erythropoietic system. During development, mesenchymal cells are characterized by its ability to invade the ECM and migrate great distances in the embryo, unlike epithelial cells (Hay 2005). For this to occur, they lose their apico-basal polarity, undergo a cytoskeleton reorganization and become bipolar (Hay 2005). As so, mesenchymal cells are loosely organized in a three-dimensional extracellular matrix and integrate the connective tissues adjacent to epithelia (Fig. 8).

It is important to note that EMT is not always characterized by the transformation of an epithelial cell into a fibroblast (Nieto 2011). In some situations where EMT is thought to have occurred, namely in fibrosis, the tissue is mostly composed by activated fibroblastic and fibrogenic cells that lead to organ failure. However, EMT does not necessarily give rise to fully differentiated mesenchymal cells. Rather, EMT is a gradual process and so intermediate phenotypes occur (Fig. 8). The concept of partial and "complete" EMT will be discussed in a further section.

The major phenotypes related with EMT will be discussed in more detail in the next section.

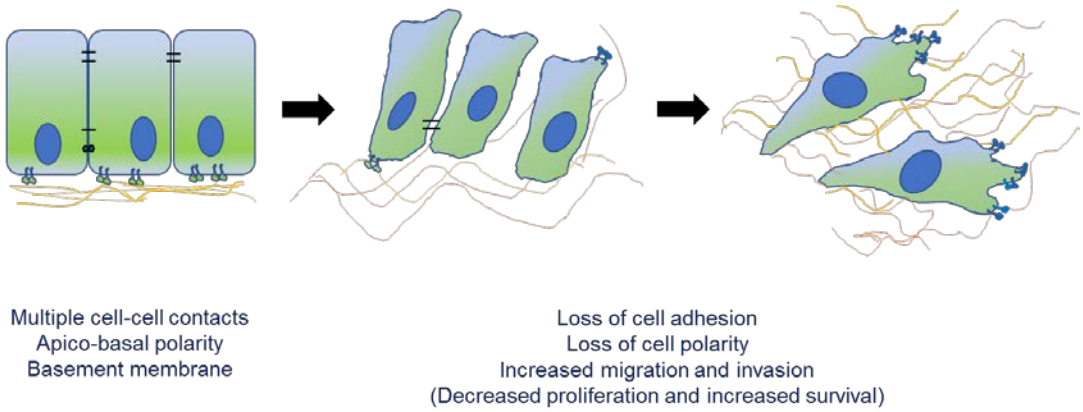

Fig. 8: Phenotypic changes that occur during epithelial to mesenchymal transition.

Cell adhesion and polarity

Cell-to-cell adhesion and cell polarity are the two major and indissociable features of cell morphology, which reflect the specialized domains that compose the plasma membrane and their association with the networks in the cytoplasm.

Cell adhesion

The epithelial phenotype is defined at the level of a group of cells and the interactions established by the cells within the group. Cell adhesion is crucial for the assembly of individual cells into the three-dimensional tissues, allowing the proper physiological function of the organ. Epithelial cells display tight junctions, adherens (also called anchoring) junctions and gap junctions. Tight junctions are impermeable junctions that seal off the intercellular space, controlling epithelia permeability, and are formed by three major groups: occludins, claudins and junctional adhesion molecules. Gap junctions, which are formed by connexins, are important for the communication between cells since they allow the direct passage of signaling molecules. These will not be further detailed in this thesis. Finally, adherens junctions are composed of cadherins, integrins, selectins among others (Pawlina and Ross 2015). Stability is a key feature of cellular junctions but they are not static and require active cellular processes. Another aspect required for maintaining tissue architecture is the association between adhesion proteins and the cytoskeleton of the cell (Vasioukhin et al. 2000). Furthermore, adhesion molecules participate in a large variety of signal transduction events that regulate cell adhesion and cell motility, cell growth, apoptosis, and specific gene regulation (Gumbiner 1996).

Adherens junctions

EMT is characterized by the disassembly of adherens junctions' complexes and by the reorganization of the actin cytoskeleton. The main component of adherens junctions are the classical cadherins. Cadherins are, in fact, one of the most important and ubiquitous proteins involved in cell-to-cell interactions. Cadherins comprise a large superfamily of membrane-associated proteins, characterized by the presence of multiple and highly conserved repeats of "extracellular cadherin domains", β-sandwich domains that adopt a similar fold to immunoglobulin domains. Cadherins can be grouped into the classical cadherins, the non-classical cadherins, which include desmosomal cadherins, and protocadherins (Paredes et al. 2012) . Classical cadherins are calcium-dependent transmembrane glycoproteins that form homophilic bonds between adjacent cells (Brasch et al. 2012). Although most biological functions are associated with homophilic interactions, classical cadherins can, in some situations, form

heterophilic interactions too (Basu et al. 2017). The extracellular cadherins domains bind calcium ions that are critical to the adhesive transbinding. Specifically, these ions contribute for the ectodomain rigidification, for defining the structure of the X-dimer interface surfaces and for strand swapping (Brasch et al. 2012). The intracellular domain of the classical cadherins interacts with the cytoskeleton through linker molecules, called catenins, to form the cytoplasmic cell-adhesion complex.

E-cadherin and N-cadherin are among the best characterized classical cadherins that have implicated in EMT (Brasch et al. 2012). In the characterization of EMT, they are generally considered markers of epithelial and mesenchymal state, respectively.

E-cadherin

E-cadherin is a transmembrane receptor that, in normal epithelia, is typically localized in clusters at the adherens junctions between adjacent cells (Quang et al. 2013). E-cadherin (which initially was termed epithelial-cadherin) was originally associated with epithelial tissues (Hatta, Okada, and Takeichi 1985) and is expressed in most of the epithelial tissues across mammalians. The cytoplasmic domain of E-cadherin binds to β-catenin (or in some situations to its homologue γ-catenin [(McCrea, Turck, and Gumbiner 1991]) and to p120 catenin. β-catenin interacts with α-catenin, which in turn anchors to the actin cytoskeleton, either directly or indirectly via actin-binding proteins α-catenin, α-actinin and vinculin (Knudsen et al. 1995; Rimm et al. 1995; Weiss et al. 1998) while p120 regulates cadherin turnover and modulates actin assembly (Davis, Ireton, and Reynolds 2003; Xiao et al. 2003) (Fig. 9).

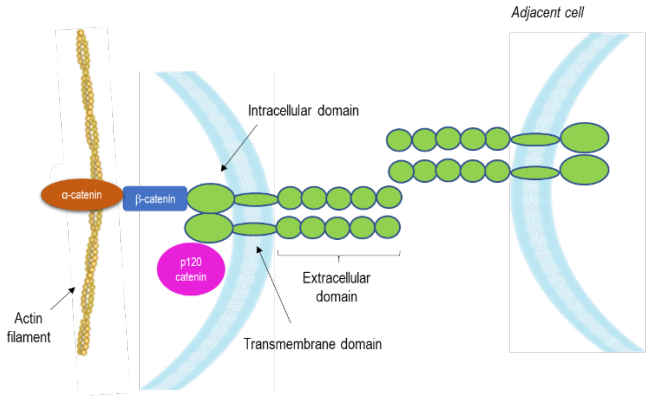

Fig. 9: E-cadherin structure and the E-cadherin-catenin complex at the junction between two neighboring epithelial cells.

The importance of E-cadherin for tissue organization and dynamics is reflected on its regulation at multiple levels. E-cadherin is encoded by the *CDH1* (cadherin 1) gene in humans and its transcription can be induced or repressed by several regulatory elements. In epithelial cells, for example, the transcription factor AP2 binds the *CDH1* promoter inducing its expression, with the retinoblastoma protein (Rb) and c-Myc as co-activators (Batsché et al. 1998). On the other hand, transcription factors associated with the EMT transcriptional program such as Snail, Slug and Zeb1/2 (described later in section "EMT Transcription Factors"), are potent negative regulators of E-cadherin (Batlle et al. 2000; Cano et al. 2000; Comijn et al. 2001; Grooteclaes and Frisch 2000; Hajra, Chen, and Fearon 2002). E-cadherin expression can also be downregulated through promoter hypermethylation, a mechanism observed in several cancer types and first described by Graff *et al.* (Graff et al. 1995). In addition to being regulated at the gene expression level, E-cadherin sub-cellular localization is key for its function. E-cadherin trafficking to and from the cell membrane is done through the endocytic and the exocytic pathways and regulated by vesicle-trafficking machinery. E-cadherin has also been shown to harbor post-translational modifications, such as phosphorylation and glycosylation. Activation of the Src tyrosine kinase, for instance, induces E-cadherin phosphorylation (presumably displacing p120) and this consequently leads to E-cadherin ubiquitination and endocytosis (Fujita et al. 2002). Furthermore, the ectodomain of E-cadherin can be N- or O-glycosylated, a modification that plays an important role on the regulation of this protein (Pinho et al. 2011). This is exemplified by the fact that the cell surface transport of the newly synthesized E-cadherin can be blocked by O-glycosylation (Zhu, Leber, and Andrews 2001).

The function of E-cadherin in several physiological processes has been investigated for a long time. E-cadherin has a central role in regulating the intercellular junction organization of keratinocytes as well as in epidermal morphogenesis (Wheelock and Jensen 1992); its crucial role was highlighted by *in vivo* experiments where *CDH1* knock-out was shown to be embryonic lethal, due to failure to form a trophectoderm epithelium (Larue et al. 1994). Loss of E-cadherin is considered a key event of EMT that triggers a major cytoskeleton organization and several signaling cascades. The role of E-cadherin in developmental EMT was investigated regarding morphogenetic events, for example in mesoderm formation, where the loss of E-cadherin-mediated cell contacts *in vitro* triggers a change in phenotype characteristic of the differentiation pathway of primitive streak mesoderm (Burdsal, Damsky, and Pedersen 1993). In cancers of epithelial origin, E-cadherin expression is frequently lost alongside tumor progression, due to several mechanisms, such as mutations, promoter methylation, transcriptional downregulation, increased endocytosis or degradation, and it is especially relevant in gastric (Corso et al. 2013; Oliveira et al. 2013) and breast tumors (Berx and Roy 2001; Corso et al. 2016). Indeed, hereditary diffuse gastric cancer is particularly associated with mutations or other alterations that lead to E-

cadherin destabilization and loss-of-function, being the underlying genetic cause of this pathology (Simões-Correia et al. 2012). As mentioned before, loss of *CDH1* expression is frequently associated with epigenetic events, such as promoter hypermethylation which have been considered a potential drug target, for example, in breast cancer (Huang, Ding, and Yang 2015). Moreover, transcriptional repression of *CDH1* by the EMT-related transcription factors is associated with the signaling induced by several growth factors and cytokines. The impact of E-cadherin in tumorigenesis is associated with the induction of migratory and invasive features, which are characteristics of EMT. Downregulation of E-cadherin in non-invasive Ras transformed cells results in a fibroblast-like phenotype and increased invasiveness when cultured in an embryonic chick heart (Navarro et al. 1991). Moreover, loss of E-cadherin-mediated cell adhesion is sufficient to promote tumor invasion *in vivo* and seems to be a rate-limiting step in the progression from adenoma to carcinoma in pancreatic carcinogenesis (Perl et al. 1998). Nonetheless, E-cadherin downregulation is not necessarily always associated with EMT. For example, when *CDH1* is knocked-down in a non-transformed mammary epithelial cell line (MCF10A), there is a disruption of the organization of the actin and tubulin cytoskeletons, together with a decreased cell adhesion to a substrate, decreased migration and upregulation of some tight junctions' proteins, but these changes were not accompanied by an EMT-associated gene expression profile, which suggests that E-cadherin downregulation is insufficient to induce an EMT (Chen et al. 2014). It is also debatable if E-cadherin can be considered an hallmark of EMT (Hollestelle et al. 2013), a controversy related to the partial EMT phenotype and cell plasticity concept that will be discussed later. The re-expression of E-cadherin in several cancer models has yielded contradictory results. On the one hand, *CDH1* transfected into highly invasive epithelial tumor cell lines of dog kidney or mouse mammary gland origin, leads to decrease in invasiveness (Vleminckx et al. 1991) and transfection into squamous carcinoma cells results in a partial reversion of their tumorigenic phenotype (Navarro et al. 1991). On the other hand, simply expressing exogenous E-cadherin in spindle carcinoma cells, which show a fibroblast-like morphology in culture is not sufficient to change this phenotype or to significantly alter the tumorigenic behavior of spindle cells, regardless of the ability to confer calcium-dependent aggregation (Navarro, Lozano, and Cano 1993). This means that E-cadherin re-expression does not always revert EMT and tumorigenicity and the differences may reside on the genetic and signaling context of the cell and their ability to organize the exogenously expressed E-cadherin into functional adherens junctions.

Other components of the adherens junction, such as α-catenin, when ablated have also been shown to be sufficient to promote precancerous hyperproliferation and sustained activation of the Ras-mitogen-activated protein kinase (MAPK) cascade in the skin (Vasioukhin et al. 2001).

Interestingly, E-cadherin plays an important role in coordinated collective migration of epithelial cells. Disruption of cell-cell junctions through β-catenin silencing leads to uncoordinated and random movement of single cells that delays the progression of the group of cells (Vedula et al. 2012). In the *Drosophila in vivo* model of collective migration, E-cadherin has a key role in orchestrating the collective cell migration namely in the direction sensing mechanism (Cai et al. 2014). Epithelial sheets can be rearranged through contractions of an actomyosin belt, constituting a form of collective migration that happens during zebrafish gastrulation (Behrndt and Salbreux et al. 2012). In this type of movement, cell adhesion is maintained and the expression landscape is not coordinated by the transcription factors (TFs) that characterize EMT. This collective migration can also happen during cancer progression (Labernadie et al. 2017).

N-cadherin

N-cadherin is a transmembrane calcium-binding protein usually associated with adherens junctions in the context of non-epithelial tissues and EMT. The sequence homology between N- and E-cadherin is high but N-cadherin forms homodimers with higher affinity (Katsamba and Carroll et al. 2009). Similarly to E-cadherin, N-cadherin intracellular domain also binds catenins, like p120 catenin, β-catenin and α-catenin, which regulate its activity, trafficking and downstream signaling (Derycke and Bracke 2004). The extracellular domain of N-cadherin forms *cis* and *trans* interactions, which are established within molecules emerging from the same membrane and with monomers on the surface of apposing cells, respectively (Bunse and Garg et al. 2013). N-cadherin is encoded by the *CDH2* (cadherin 2) gene and some transcriptional regulators have already been described (Derycke and Bracke 2004). During gastrulation, the transcription of the *CDH2* homologue in *Drosophila*, is initiated by the transcription factor Twist, while Snail is also required for its complete upregulation (Oda, Tsukita, and Takeichi 1998). In a pathological context, Twist is necessary for N-cadherin expression in prostate cancer cells (Alexander et al. 2006). Interestingly, this study also suggests that Twist-induced N-cadherin expression is dependent on integrin-β1–mediated cell adhesion to fibronectin (Alexander et al. 2006). On the other hand, much more is known concerning the regulation of N-cadherin and its associated molecules, through post-translational modifications. Like E-cadherin, N-cadherin is a glycoprotein and this post-translational modification confers a fine-tuning regulation of its functional role depending on the residue and the cell context. For example, inhibition or deletion of N-cadherin N-glycosylation leads to increased stabilization of cell-cell contacts, intracellular signaling mediated by cell-cell adhesion and reduced cell migration, with no impact on its expression at the membrane (Guo et al. 2009). In glioma cells, elimination of specific N-glycans promotes proteasomal degradation of N-cadherin and inhibits cadherin-mediated cell–cell

adhesion and promotes cell migration (Xu et al. 2017). Another post-translational modification important for regulating N-cadherin function is phosphorylation. For example, N-cadherin, which participates in neuron communication, as it undergoes a conformational change upon depolarization in the synaptic junctional adhesion in the central nervous system (Tanaka et al. 2000), was shown to be phosphorylated by the serine/threonine kinase protein kinase D1 which increases its membrane localization and promotes functional synapse formation (Cen et al. 2017).

N-cadherin-triggered homophilic adhesion of cells to a substrate is characterized by the induction of lamellipodia protrusions and a cytoskeleton reorganization. The organization of cadherin-catenin complexes and actin filaments in cadherin adhesions is dependent on membrane recruitment of p120, which contributes for a Rac1-dependent extension and maturation of cadherin-mediated contacts (Gavard et al. 2004). In fact, cadherins are associated with several signaling pathways, such as Rho-family GTPases, through catenins. Rho-family GTPases, including RhoA, Rac1, and Cdc42, are molecular switches - cycling between an active GTP-bound state, which allows association with an effector protein, and an inactive GDP-bound state - and have emerged as key regulators of cadherin-mediated cell-cell adhesion, being their differential activation determinant for the resulting cell phenotype (reviewed in Braga 2000). Furthermore, N-cadherin interacts with fibroblast growth factor (FGF) receptor, preventing its ligand-induced internalization, being this stabilization essential for FGF-2 sustained activation of the MAPK-ERK pathway, which leads to MMP-9 gene transcription and cellular invasion in cancer cells (Suyama et al. 2002). The formation of N-cadherin-induced lamellipodia is also associated with an activation of the PI3K pathway (Gavard et al. 2004), a pathway that also mediates cell survival (Li, Satyamoorthy, and Herlyn 2001; Tran et al. 2002; Zuo et al. 2013). N-cadherin also mediates the expression of the anti-apoptotic cell survival protein Bcl-2 through the PI3K/Akt pathway in human prostate carcinoma cell lines (Tran et al. 2002). Finally, glial cell line-derived neurotrophic factor exerts a neuro-protective effect on dopaminergic neurons via N-cadherin and the PI3K/Akt pathway (Zuo et al. 2013).

N-cadherin was originally described in more detail in the nervous tissue - hence named as neural cadherin - and during development, where its expression is detected upon gastrulation (Hatta, Okada, and Takeichi 1985; Kohei Hatta and Takeichi 1986). In a cell biology perspective, N-cadherin plays a major role in migration. Exogenous expression of N-cadherin in epithelial cells induces a change in their morphology and invasive properties. In model of squamous cell carcinoma, N-cadherin is associated with a scattered morphology in cultured cells and with invasion in tumor biopsies. Besides, when N-cadherin is expressed in squamous epithelial cells, it induces a more scattered and less adhesive phenotype, resembling features of EMT (Islam et al. 1996). Similar observations were made in breast cancer cell lines: N-cadherin promotes migration and invasion *in vitro* and metastasis *in vivo* (Hazan et al. 2000; Nieman et al. 1999).

Concerning metastasis, N-cadherin was shown to enhance metastatic potential to the lungs of mammary epithelial cells expressing the polyoma virus middle T antigen (Hulit et al. 2007).

Cadherin switching

Frequently during EMT, N-cadherin expression becomes upregulated while E-cadherin is downregulated, a phenomenon termed 'cadherin switching'. It may also involve alterations in other cadherins, such as P-cadherin. This observation has been reported during development, for example in gastrulation (Oda, Tsukita, and Takeichi 1998) or in kidney morphogenesis (Dahl et al. 2002), as well as in several cancer types, like prostate cancer, breast cancer and pancreatic cancer (reviewed in Wheelock et al. 2008). In squamous cell carcinoma it has been reported an inverse correlation of the expression levels of N-cadherin and P-cadherin versus E-cadherin: in fact, when epithelial cells are transfected with N-cadherin, E-cadherin and P-cadherin become downregulated (Islam et al. 1996). This may suggest a reciprocal or coordinated regulation of both E-cadherin and N-cadherin. In fact, a collective downregulation of adherens junction components (E-cadherin, α-catenin, β-catenin and p120-catenin) has been observed in prostatic adenocarcinomas patients' samples (Kallakury, Sheehan, and Ross 2001). During TGF-β-induced EMT in mammary cells, cadherin switching seems to be essential for increased cell motility, but not for changes in morphology (Maeda, Johnson, and Wheelock 2005).

Tight junctions

Other important structures that constitute the apical junctional complex, together with adherens junctions, and that regulates cell communication and cell polarity are tight junctions. In epithelial cells, tight junctions localize closest to the apical side and divide the cell surface into two functionally and biochemically distinct regions, which face either one of the two physiological compartments. Tight junctions work as selective permeability barriers that regulate the paracellular route for the movement of ions and solutes (Farquhar and Palade 1963; Gumbiner 1987). They are composed of the transmembrane proteins claudin and occludin and cytoplasmic proteins that provide scaffolding, such as zonula occludens (ZO-) 1, 2 and 3 and several other cytoskeleton-associated proteins. Occludins have a major regulatory role for assembly/disassembly and function of tight junctions (Yu et al. 2005). These structures are very diverse and plastic since their permeability and signaling properties vary from tissue to tissue, their composition changes and they are subject to rapid regulation. For example, tight junctions' stability can be regulated through post-translational modifications. Occludins can be phosphorylated in a process mediated by the Src-family tyrosine kinases c-Yes and c-Src, Rho

kinase, protein kinases C, casein kinase 1, VEGF or PDGF (Dörfel and Huber 2012). Phosphorylation by these kinases affects occludin half-life, localization and interaction with other tight junctions' proteins (Dörfel and Huber 2012).

As a key part of their communication role, tight junctions are intimately connected to the cytoskeleton and activate signaling cascades. One of the main components of the signaling pathways activated downstream of tight junctions are Rho family GTPases (Zihni, Balda, and Matter 2014), for example, the guanine nucleotide exchange factor (GEF) for RhoA, GEF-H1 (also known as ARHGEF2) that regulates paracellular permeability (Benais-Pont et al. 2003). Moreover, GEF-H1 is recruited to tight junctions by binding to the junctional adaptor cingulin, which results in the inhibition of GEF-H1, with consequential inhibition of various RhoA-driven processes, such as cell cycle progression and gene expression. Interestingly, cingulin expression increases with increasing cell confluence and so, this mechanism may provide a link for the regulation of RhoA signaling and cell cycle by cell confluence (Aijaz et al. 2005; Benais-Pont et al. 2003). Furthermore, the Cdc42 effectors PAK4 and partitioning defective (Par) 6B are required for tight junction formation and junctional maturation (Wallace et al. 2010).

Tight junctions' dysregulation is associated with EMT. Using a luminescence-based approach to unravel the interactome for TGF-β signaling, occludin and Par6 were identified as binding to TGF-β receptors. Upon TGF-β treatment, TGF-β type II receptor was found to be recruited to tight junctions, phosphorylating Par6 and inducing RhoA degradation in a Smurf1-dependent manner. The phosphorylation of Par6 is required for TGF-β-induced EMT and interferes with the cortical actin cytoskeleton and ultimately contributes to tight junction disassembly (Barrios-Rodiles et al. 2005; Ozdamar et al. 2005). In another EMT model, where Raf constitutively active expression induces transformation, cells lose their epithelial phenotype and E-cadherin expression at the membrane, while the tight junctions become disassembled, a phenotype that can be rescued by restoring occludin expression (Li and Mrsny 2000). Specifically, Raf1 induces the upregulation of the transcriptional factor Slug, which inhibits the expression of occludin (Wang et al. 2006). In fact, Snail and Slug, two transcription factors that play a major role in EMT, when overexpressed disrupt tight junctions and have been shown to repress the expression of claudins and occludin (Ikenouchi et al. 2003; Martínez-Estrada et al. 2006). The role of tight junctions on EMT is especially evident when cells are cultured under confluent conditions *in vitro*. ZO-1 is able to bind and regulate the intracellular localization of transcription factor named ZO-1-associated nucleic acid-binding protein (ZONAB). The binding of ZO-1 to ZONAB occurs in confluent culture conditions and sequesters the transcription factor within the tight junctions, preventing its nuclear translocation where it represses Erb2 expression, a tyrosine kinase co-receptor important for epithelial differentiation (Balda and Matter 2000). ZONAB also regulates epithelial cell proliferation and cell density through binding and inhibition of the nuclear function

of cell division kinase-4 (CDK4) in confluent conditions (Balda, Garrett, and Matter 2003). Since ZO-1 loss of activity has been associated with EMT, ZO-1 has actually been considered an EMT marker (Huang, Guilford, and Thiery 2012; Zeisberg and Neilson 2009).

The importance of tight junctions in EMT is dependent on the cell line, since there are cell lines that, under normal culture conditions, do not form tight junctions, as exemplified by MCF10A cells (used on chapter 3.2 of the "results" section) (Underwood et al. 2006).

Cell polarity

Cell polarity is a major entity in cell morphology that regulates cell behavior, namely cell communication, cell identity and cell movement. Cell polarity is a key determinant during cell cycle and for the establishment of the division plane. It also determines the direction of growth and the formation of cell protrusions.

Polarized epithelial cells form a continuous cell layer, creating a barrier that define the shape of several organs. This confers protection and, at the same time, allows diverse polarized activities including absorption, secretion, transcellular transport and sensation.

Cell polarity is initiated by the recognition of a spatial cue, such as cell-to-cell adhesion in the case of epithelial cells, which is decoded by membrane complexes and triggers downstream signaling events. This causes an assembly of the cytoskeleton, redistribution of the microtubules and of the secretory pathway components. Epithelial cell polarity requires both cell–cell and cell–ECM adhesion, in order to establish structurally and functionally distinct apical and basal–lateral membrane domains (Drubin and Nelson 1996). Cell polarity is coordinated by three main protein complexes, the so called 'polarity complexes' - Crumbs, Par and Scribble complexes - and their interactions with cell-cell adhesion receptors, such as tight junctions and adherens junctions (forming the apical junctional complexes) and Rho family GTPases (Ngok, Lin, and Anastasiadis 2014). E-cadherin and catenins play a role in cell polarity. For example, catenin downregulation leads to reduced cell polarity (Vasioukhin et al. 2001). However, the role of these adherens junctions' proteins in cell polarity seems to be dependent on the signaling context, as exemplified by mammary epithelial cells, in which E-cadherin downregulated cells still retained cell polarity, based on the apical ZO-1 localization (Chen et al. 2014). The establishment, but not maintenance, of apical-basal polarity was also compromised in kidney epithelial cells after E-cadherin knock-down (Capaldo and Macara 2007).

Cell polarity has been shown to participate in tumorigenesis. For example, in human colorectal tumors, the decreased expression of DGL1 (discs large MAGUK scaffold protein 1) and SCRIB (scribbled planar cell polarity protein), two genes that have been considered tumor suppressors in

Drosophila and are commonly downregulated in cancer, is associated with the lack of epithelial cell polarity and a disorganized tissue architecture (Gardiol et al. 2006).

EMT is frequently associated with changes in epithelial cell polarity and several polarity regulators have been linked with EMT. In particular, Zeb1, an EMT-associated transcription factor, is necessary for loss of basal-apical cell polarity during EMT by directly repressing the cell polarity genes Hugl2 (or Lgl2), Crumbs3, and Pals1-associated tight junction protein through binding to their promoters in colorectal and breast cancers (Aigner et al. 2007; Spaderna et al. 2008). Conversely, Scribble, another polarity complex, mediates the expression of Zeb via MAPK-ERK signaling and Fra1 (Elsum, Martin, and Humbert 2013). Par3, a protein from the Par complex, is downregulated after TGF-β treatment together with other characteristic EMT features, which leads to the redistribution of the Par6–α protein kinase C and complex from the membrane to the cytoplasm and the subsequent disorganization of the Par complex, leading to a loss of polarity (Wang et al. 2008).

Migration and invasion

Increased migration is a key feature of EMT during development, wound healing response and fibrosis and in cancer. Cell motility that characterizes EMT in the embryo has been subject of study since the 80s (Hay 1989). For example, contractile actomyosin rings drive cell movements during zebrafish gastrulation (Behrndt and Salbreux et al. 2012). Besides, FAK was shown to regulate Snail1-dependent EMT in embryonic cells (Li et al. 2011).

The study of cell migration and invasion is of particular interest in cancer research as it is a major determinant for metastatic progression. In this setting, EMT can be regarded as one of the ways by which cancer cells are capable of migrating and invading the adjacent tissue, the first step in cancer progression towards the production of metastasis. The interstitial migration and invasion program of mesenchymal cells in cancer is characterized by single cell movement, prominent protrusions and spindle-shaped morphology, strong adhesion to ECM and proteolytic tissue remodeling (Friedl and Wolf 2003). Increased cell migration and invasion reflect several changes in the protein expression and signaling landscape that characterize EMT and so, several of the proteins that regulate mesenchymal migration are used as markers of EMT, including N-cadherin, fibronectin, MMPs and vimentin.

Fibronectin is one of the most commonly used EMT markers. Fibronectin characterizes the breast cancer ECM (Helleman et al. 2008), while in mammary epithelial cells, the coating of cell culture plates with fibronectin not only induces an increased migratory behavior, but also the upregulation of fibronectin as well as the expression of several proteins associated with EMT such as N-

cadherin, vimentin, MMP-2 and Snail (Park and Schwarzbauer 2014). As previously mentioned, fibronectin can bind several integrin combinations, such as α5β1 (Humphries, Byron, and Humphries 2006), being the integrin binding domain on fibronectin sufficient to induce the previously mentioned effects on EMT protein expression (Park and Schwarzbauer 2014). Fibronectin was also shown to lead to a TGF-β pathway activation and endogenous production which is required for the EMT induction (Park and Schwarzbauer 2014). Moreover, using a computational model, it was shown that ECM density also promotes EMT by weakening of cell–cell adhesions (Kumar, Das, and Sen 2014).

MMPs are also commonly used as a marker of EMT although the expression of individual MMPs depends on the tissue type. The MMP-3 (or stromelysin-1), for example, changes the phenotype of mammary epithelial cells to invasive mesenchymal-like cells in a process mediated by E-cadherin cleavage and β-catenin redistribution (Lochter et al. 1997; Sternlicht et al. 1999). Interestingly, MMPs from the microenvironment also seem to directly induce EMT in epithelial cells, as shown in a mouse mammary carcinoma model, where MMP-9 mediates the proteolytic activation of latent TGF-β, after docking to the hyaluronan receptor CD44 at the membrane. This promotes tumor invasion and angiogenesis (Yu and Stamenkovic 2000).

Vimentin is an intermediate filament protein, generally expressed in mesenchymal cells, which forms a cytoskeleton network for the nucleus hedge to the membrane and provides mechanical resistance to cells (Chang and Goldman 2004). Importantly, vimentin is involved in cell migration, for example through interaction with filamin A and mediating integrin-β1-regulated cell spreading (Eckes et al. 1998; Kim et al. 2010). Vimentin filaments also interact with signaling proteins, such as phosphorylated ERK1/2 in a calcium dependent manner, protecting the kinase from dephosphorylation (Perlson et al. 2005, 2006). Vimentin expression is sufficient to induce the changes in cell shape and motility typical of the EMT (Mendez, Kojima, and Goldman 2010). Specifically, vimentin is necessary for invadopodia elongation (Schoumacher et al. 2010) and it is also required for focal adhesions formation, actin and microtubules cytoskeleton organization and directional migration in breast cancer cell lines. In fact, in a breast cancer cell line, vimentin expression results in Slug and integrin-β1 expression and E-cadherin downregulation (Liu et al. 2015).

Within a tissue, cell migration is dependent on a physicochemical balance between cell deformability and physical constraints. An interesting aspect of cancer cell migration, that is especially relevant when individual cells move through very tight interstitial spaces during metastasis formation, is the nucleus translocation and deformation. This process is regulated by the accumulation of non-muscle myosin IIB in the back of the nucleus (Thomas et al. 2015). Physical stress during migration can result in nuclear rupture and DNA damage. Laminins A/C

are important in protecting against nuclear envelope rupture and membrane remodeling proteins like ESCR-III (endosomal sorting complexes required for transport III) are involved in nuclear damage repair (Denais and Gilbert et al. 2016). Aggressive breast cancer cell lines show deformed nucleus architecture and have lower levels of laminin A/C and the downregulation of these proteins is also correlates with disease recurrence in colon cancer patients (Belt et al. 2011; Hutchison 2014; Matsumoto et al. 2015). These data raise an interesting question: does nuclear stress have an active role in cancer progression by promoting genomic instability? (Denais and Gilbert et al. 2016).

It is important to reinforce that cancer cell movement can occur in the absence of EMT (Friedl and Alexander 2011). It was observed, using intravital imaging in live mice that breast cancer cells can switch from a collective type of movement to individual cell migration, in a process regulated by TGF-β signaling. Single cell movement allowed cells to enter the bloodstream, whereas collective invasion seems can mediate lymphatic dissemination (Giampieri et al. 2009).

Proliferation and survival

The relationship between EMT and proliferation rate has been subject of debate and it seems highly dependent on the signaling context of the cells. In general, EMT is associated with reduced proliferation, both in the context of development or cancer associated EMT.

The coordination of cell cycle during development is crucial for morphogenesis and cell fate determination. The analysis of the cell cycle length in mesodermal and ectodermal cells of rat embryos during gastrulation showed that the cells of the primitive streak divided less often compared to epiblast cells before ingression (Mac Auley, Werb, and Mirkes 1993). In the *Drosophila* embryo, the cell cycle regulator Tribbles delays mitosis by downregulating String, a CDC25 mitotic activator, thus allowing gastrulation to be completed before cell division takes place (Mata et al. 2000). When a timed mitotic block is missing in cells undergoing EMT, there is a developmental failure of embryos (Mata et al. 2000).

One of the most commonly used EMT inducers, TGF-β, has a dual role in proliferation in cancer: it can repress cell division or promote tumor growth, depending on the tissue (Derynck, Akhurst, and Balmain 2001; Massagué 2008). Specifically, in normal epithelial cells, TGF-β suppresses proliferation through Rb, causing an arrest at the G1 phase of the cell cycle and this suppression of proliferation can happen in the context of EMT (Laiho et al. 1990; Peinado, Quintanilla, and Cano 2003). Tumor cells can avoid growth inhibitory activities of TGF-β, through inactivation of certain components of the pathway due to genetic alterations or transcriptional silencing; however, they retain sensibility to other TGF-β responses due to different thresholds of signaling

(Derynck, Akhurst, and Balmain 2001; Massagué 2008). On the other hand, TGF-β can also trigger apoptosis (Derynck, Akhurst, and Balmain 2001; Massagué 2008) but in hepatocytes it has been shown that EMT confer resistance to the apoptotic effects of TGF-β (Valdés et al. 2002).

An interesting study helped to clarify the compatibility (or lack of it) of cellular proliferation and EMT (Comaills et al. 2016). Cells that persist proliferating after EMT induction by TGF-β treatment present mitotic defects and genomic abnormalities. The defects in mitosis are due to failed cytokinesis and associated with suppression of nuclear envelope proteins implicated in mitotic regulation such as laminB1. Besides, EMT-induced mitotic defects are reversible, but the inherited genomic instability persists and promotes tumorigenic phenotypes (Comaills et al. 2016).

Using an inducible cell line model of EMT (non-small cell lung cancer cells with inducible Twist and Snail) to investigate how induction of the transition alters signaling networks and requirements for proliferation, Salt *et al.* reported that proliferation is affected during EMT, particularly in serum free conditions due to disruption of the neuregulin 1, Erb-B2 receptor tyrosine kinase 3 and PI3K-AKT pathway (Salt, Bandyopadhyay, and McCormick 2014).

Mechanistically, Snail was shown to induce cell cycle arrest in G1, through several cell cycle regulators of the early to late G1 transition and of the G1/S checkpoint. Specifically, Snail represses cyclin D2 transcription leading to a maintenance of the low levels of cyclins D and blockage the G1/S transition by maintaining high levels of p21 (Vega et al. 2004). Moreover, Snail also promotes cell survival under serum depletion conditions through the MAPK and PI3K pathways (Vega et al. 2004). Like Snail, Slug activation also reduces the proliferation, concomitantly with decreased ERK activity and decreased Rb phosphorylation (Turner et al. 2006). Besides, Zeb2 binds cyclin D1 gene, repressing its transcription which leads to accumulation of cells in the G1 phase of cell cycle (Mejlvang et al. 2007). Yet, Twist was shown to promote tumor cell proliferation (Ansieau et al. 2008; Qian et al. 2013; Shiota et al. 2008). In gastric cell lines, Twist induces FoxM1 expression, which plays a key role in cell cycle progression (Qian et al. 2013), while its downregulation induces G1 arrest and decreases cell proliferation (Shiota et al. 2008). Moreover, Twist1 and Twist 2 suppress oncogene-induced premature senescence in cancer cells, cooperating with Ras for malignant transformation and disrupting both p53 and Rb pathways (Ansieau et al. 2008). However, another study in an *in vivo* skin tumor model showed that induction of Twist1 is associated with EMT and reduced tumor cell proliferation and that turning off Twist1 at distant sites promoted metastatic growth (Tsai et al. 2012).

Concluding, EMT results from the alteration of multiple components of cellular phenotype, although the degree of these changes varies according to the cellular context. Specification of cells that will undergo EMT occurs through coordination of cell–cell, cell-ECM and soluble signals with intracellular mediators.

1.3.2 - EMT regulation

How is EMT induced? How are the changes in cellular phenotype coordinated?

In adult tissues, EMT can be triggered by the composition, structure of the ECM components and its remodeling (Hay 1990; Masszi et al. 2004; Radisky et al. 2005), nutrient and oxygen conditions (Imai et al. 2003; Lv et al. 2011), as well as soluble growth factors or cytokines, including EGF, HGF, FGF, and TGF-β (Thiery et al. 2009).

EMT is characterized by the activation of a transcriptional program that triggers and is responsible for changes in major cell structures and functions. Actually, EMT is a program regulated at different levels, namely chromatin changes, miRNAs, transcription, splicing, post-translationally (Nieto et al. 2016). For example, the miR200 family, highly expressed in epithelial tissues and involved in establishing epithelial lineages in early development, is one of the most studied mechanisms of regulation of EMT, being dysregulated in many epithelial cancers (Brabletz and Brabletz 2010). This family of microRNAs is induced by several EMT stimuli, targeting and downregulating EMT transcription factors such as Zeb 1 and Zeb2 (Korpal et al. 2008; Park et al. 2008). *Zeb* is a particular target of miR200 since its gene sequence is highly enriched in binding sites for this specific family of miRs. Besides, Zeb also regulates the transcription of miR200 family (Brabletz and Brabletz 2010). Polycomb repressive complexes (PRCs), which silence transcription by modifying histones and recruiting a variety of additional repressors also participate in the induction of EMT (Tam and Weinberg 2013).

Nowadays, the transition is considered a dynamic process that reflects cellular plasticity. In many examples, either from studies in cell culture to *in vivo* and from cancer and fibrosis to wound healing and developmental processes, cells undergoing EMT exhibit several intermediate phases, sometimes called “stable and metastable states”, and do not reach a “fully mesenchymal” phenotype. These states are not characterized by a universal gene expression pattern (Nieto et al. 2016).

Next, I will introduce the inducers and effectors of the EMT program. Concerning the EMT effector molecules, I will be focusing on the regulation at the transcriptional levels by transcription factors.

Signals and pathways that induce EMT

Many growth factors and cytokines have been described as EMT inducers including TGF-β, TNF-α, BMP, EGF, FGF, PDGF, Wnt-β-catenin and Notch (Thiery et al. 2009). Among the cytokines that are capable of triggering EMT, TGF-β and TNF-α are the most commonly used for *in vitro* experiments. The specific roles of these molecules will be mentioned through-out the following sections of this thesis. Besides, the contact with the surrounding matrix can also trigger or be required for EMT. For example, the composition and structure of the ECM can regulate EMT in a process mediated by integrins (Hay 1990; Masszi et al. 2004; Radisky et al. 2005). The extracellular environment can also modulate the phenotype of cells through the availability of oxygen and nutrients, such as glucose (Lv et al. 2011). Hypoxia or HIF can induce the expression of EMT effectors such as Twist, Snail and Zeb (Evans et al. 2007; Yang et al. 2008). Moreover, metabolites namely succinate, fumarate and 2-HG have been shown to lead to EMT when accumulated intracellularly (Colvin et al. 2016; Sciacovelli et al. 2016; Tseng et al. 2018). These signals have been described as EMT inducers during embryonic development, fibrosis and cancer.

Concerning cancer, besides the previously mentioned cues, common oncogenic pathways, such as those associated with HRas, ErbB2 or mutant p53, can promote EMT in association with other pathways (Adorno et al. 2009; Jenndahl, Isakson, and Baeckström 2005; Oft, Akhurst, and Balmain 2002; Peinado, Quintanilla, and Cano 2003). EMT-triggering signals often come from the tumor stroma, whose components, like fibroblasts, endothelial cells and immune cells, can secrete several types of cytokines, growth factors or proteins like MMPs, which can induce or mediate the acquisition of mesenchymal properties by cancer cells (Bussard et al. 2016). Besides, EMT inducers can work as paracrine and autocrine signals (Scheel et al. 2011).

The signals that induce EMT can be very diverse, cell- or tissue-specific. Cells can indeed display different sensitivities to the same signals and their integration can be achieved differently, leading to distinct outcomes. Besides, many of these signals trigger a local response that is also highly dependent on the cell sensitivity. Finally, in response to these stimuli, several signaling pathways become activated, being EMT the result of a complex regulatory crosstalk inside the cell (Oft, Akhurst, and Balmain 2002).

EMT stimuli converge in the activation of common transcription factors, which will be described in the next section.

EMT transcription factors

The changes that epithelial cells undergo when becoming more mesenchymal are orchestrated by key factors that are commonly designated as EMT TFs. The idea of a master regulator that controls the migration machinery during EMT had already been put forward in the early days of EMT by E. Hay (Hay 1989). The role of these EMT TFs goes from gastrulation in flatworms, like planarians (Abnave et al., 2017) to roundworms like *Caenorhabditis elegans* (Manzanares, Locascio, and Nieto 2001), insects like *Drosophila melanogaster* (Grau, Carteret, and Simpson 1984; Simpson 1983), vertebrates like *Xenopus* (Sargent and Bennett 1990), mouse (Smith, del Amo, and Gridley 1992) and human (Rhim et al. 1997). In fact, an interesting study using planarians showed that the EMT transcription factor homologues of Snail-1, Snail-2 and Zeb-1, together with a matrix metalloprotease and β-integrin, are essential for pluripotent adult stem cell and progeny migration in wound repair (Abnave et al., 2017).

The contribution of these TFs to EMT is context-dependent. For example, when assessing the dependency of metastasis formation on EMT, different groups, using a pancreatic cancer mouse model, obtained distinct results depending on the EMT TFs used, suggesting functional differences of EMT TFs (Krebs et al. 2017; Zheng et al. 2015). This is matter of debate nowadays in the EMT field and further research is needed. Some of the genes regulated by the EMT TFs are shared and EMT TFs can regulate each other, creating a complex network. For example, E-cadherin is a target of both Snail and Zeb1 (Batlle et al. 2000; Cano et al. 2000; Grooteclaes and Frisch 2000) and Snail can actually promote Zeb1 expression (Guaita et al. 2002). Finally, these TFs have pleiotropic functions and much is still unknown about the genetic programs that they regulate (Goossens et al. 2017).

There are several TFs described as tightly associated with EMT (reviewed in Zheng and Kang 2013). In this thesis, I will focus on the most studied EMT TFs that belong to the Snail, Twist and Zeb families and on their role in cancer-associated EMT.

Snail family

Snail family belongs to the larger Snail superfamily, which also includes the Scratch family (Manzanares, Locascio, and Nieto 2001). Snail is a family of zinc-finger transcription factors, characterized by a highly conserved carboxyl (C)-terminal containing 4 to 6 zinc-fingers and a much less conserved amino (N)-terminal where elements that modulate Snail transcriptional activity bind (Nieto 2002). In mouse and human, Snail encodes a four zinc-finger protein with 85.5% homology between them (Paznekas et al. 1999). In vertebrates, Snail family contains 3 members: Snai1 (Snail), Snai2 (Slug) and Snai3 (Smuc). *Snail* and *Slug* are key genes during

EMT in several cellular contexts, while the function of *Smuc* is less characterized (De Herreros et al. 2010) and therefore, it will not be described any further. *Snail* genes have been suggested to act primarily as survival factors and inducers of cell migration, rather than as inducers of EMT or cell fate (Barrallo-Gimeno and Nieto 2005).

Snail

Snail was first identified in *Drosophila melanogaster* as a gene necessary for proper mesoderm formation (Grau, Carteret, and Simpson 1984; Simpson 1983) and encodes a protein with five zinc-fingers suggesting, at that time, a DNA-binding function (Boulay, Dennefeld, and Alberga 1987). It was later proposed that since Snail is expressed in multiple germ layers during development, its role is not limited to mesoderm formation (Alberga et al. 1991). Snail research was further expanded to vertebrates with the successful cloning of the *Xenopus* (Sargent and Bennett 1990) and the mouse *Snail* gene (Nieto et al. 1992; Smith, del Amo, and Gridley 1992), among others. In humans, *SNAIL* was mapped together with its pseudogene *SNAI1P1* (Paznekas et al. 1999; Twigg and Wilkie 1999). In the early studies, Snail was described to be a transcriptional repressor of genes important for development (Gray, Szymanski, and Levine 1994; Ip et al. 1992; Kosman et al. 1991; Leptin 1991).

Snail binds to a consensus sequence composed of 6 amino acids – CAGGTG -, a motif similar to the E box (Mauhin et al. 1993; Prokop et al. 2013). The most well-studied target of Snail is *CDH1* that encodes E-cadherin, an adhesion protein with a central role in EMT (see "E-cadherin" section). In 1998, it was first shown that *Drosophila* embryos mutant for *Snail* are associated with E-cadherin downregulation (Oda, Tsukita, and Takeichi 1998). It was later proven that Snail indeed binds to the *CDH1* promoter E-boxes and represses its transcription, which results in the disassembly of adherens junctions (Batlle et al. 2000; Cano et al. 2000). In tumor cell lines, this repression was shown to be dependent on the PRC2, which is recruited by Snail to the *CDH1* promoter (Herranz et al. 2008). Recently, it has been shown that Snail binds the long non-coding RNA HOTAIR (for HOX transcript antisense intergenic RNA) recruiting EZH2 (enhancer of zeste homolog 2), member of the PRC2, to specific genomic sites (Battistelli et al. 2017). We now know that Snail family members can positively or negatively regulate transcription. In a breast cancer cell line, Snail expression was shown to lead to the differential expression of 7602 genes, while an interactome analysis revealed Snail as an over-connected protein (Mezencev et al. 2016). Snail regulates tight junctions, for example by transcriptionally repressing claudins (claudin-3, claudin-4 and claudin7) and occludin (Ikenouchi et al. 2003), while in collaboration with EGR1 and SP1, it directly activates transcription of *ZEB1* and *MMP-9* induced by the tumor promoter 12-O-tetradecanoyl-phorbol 13-acetate (Wu et al. 2017). Snail also has been shown to

bind to target genes implicated in epithelial differentiation and signaling such as *PTEN*, *FOXA* family members, *Rab25* and *keratin 18* (De Craene et al. 2005; Jägle et al. 2017; Escrivà et al. 2008). Snail regulates splicing, particularly repressing ESRP1 expression by binding directly to the E-box structure in the promoter of ESRP1, thus promoting CD44 isoform switching during EMT (Reinke, Xu, and Cheng 2012). Importantly, Snail binds directly to the promoter and is able to regulate the expression of several metabolic enzymes such as PFKP, FBP1 and cytochrome c oxidase subunits (De Craene et al. 2005; Dong et al. 2013; Kim et al. 2017; Lee et al. 2012).

From the description above, it becomes clear that Snail targets are related to EMT. In fact, Snail was first implicated in EMT through the work done with mutant forms of *Snail* in model organisms. *Drosophila Snail* mutants are characterized by defects in mesoderm formation, specifically the absence of mesenchymal cells (Leptin and Grunewald 1990). Similarly, mice embryos that express a mutated form of *Snail* show a retention of epithelial characteristics and maintenance of E-cadherin expression in mesoderm, pointing Snail as a key regulator of EMT during development. These mice die early in gestation due to defects in gastrulation and mesoderm formation (Carver et al. 2001).

Cano *et al*. demonstrated that Snail overexpression in canine kidney epithelial cells results in a change to a fibroblast-like morphology, with long extensions, downregulation of E-cadherin and desmoplakins, upregulation of vimentin and fibronectin, increased migration and invasion (Cano et al. 2000). In colon cancer cells, the inducible Snail expression causes a change in cellular morphology, decreased aggregation, increased invasion, consistent with EMT (De Craene et al. 2005). On the other hand, knock-down of *SNAIL* has been shown to reverse EMT, at least partially, and the invasiveness phenotype of tumor cells (Olmeda et al. 2006; Wang et al. 2013; Wu et al. 2009). Taken together, these results suggest that Snail expression is sufficient to induce EMT.

Interestingly, Snail also regulates normal mesenchymal cell function, since in fibroblasts, Snail silencing impairs MMP-14-dependent invasive activity (Rowe et al. 2009). Snail can also have other functions, such as resistance to cell death (Kajita, McClinic, and Wade 2004), cell-cycle progression and survival (Vega et al. 2004), as well as the prevention from radiation-induced apoptosis, through regulation of PTEN and the Akt pathway (Escrivà et al. 2008).

How is Snail induced and regulated? Snail-inducing stimuli and signaling pathways include TGF-β, EGF, Notch, Wnt, ROS and hypoxia (Imai et al. 2003; Liu et al. 2014; Peinado, Quintanilla, and Cano 2003; Sahlgren et al. 2008; Yook et al. 2006). TGF-β was the first cytokine shown to induce Snail, in kidney epithelial cells, in a process mediated by the MAPK pathway (Peinado, Quintanilla, and Cano 2003). Later, it was shown that Smads, which are induced upon TGF-β

treatment, bind *Snail* promoter together with HMGA2 (high mobility group A2) leading to EMT (Thuault et al. 2008). Moreover, Snail and Smads form a transcriptional repressor complex that downregulates the expression of several epithelial-associated proteins such as E-cadherin and Coxsackie- and adenovirus receptor, a tight-junction-associated cell adhesion molecule (Vincent et al. 2009). Snail is also crucial for TNF-α mediated EMT (Wang et al. 2013) and other inflammatory cytokines, such as interleukine-1β, which have been shown to induce EMT and enhance Snail binding at the chromatin level (St. John et al. 2009). Another important pathway for development and is also activated under hypoxia conditions is the Notch pathway, which controls Snail expression through two distinct mechanisms: binding to the Snail promoter and through a HIF-1α mediated lysyl oxidase upregulation, that stabilizes the Snail protein (Sahlgren et al. 2008). Several growth factors trigger signaling cascades that upregulate Snail, for example the FGF receptor 1 (Ciruna and Rossant 2001) or EGF receptor (Lee et al. 2008; Lu et al. 2003). Oncogenic HRas signaling, through the MAPK and the PI3K pathways, and Akt signaling also induce Snail promoter (Grille et al. 2003; Peinado, Quintanilla, and Cano 2003). Interestingly, Snail seems to directly regulate itself by binding to its own promoter, creating a negative loop (Mauhin et al. 1993; Peiró et al. 2006). In several cases, Snail expression is increased by stabilization of its protein levels and cellular location, through GSK-3β-mediated phosphorylation (Zhou et al. 2004). In human embryonic kidney (HEK) 293 cells overexpressing Snail cells, the cytokine TNF-α promotes Snail stabilization via NF-kB (Wu et al. 2009), while in colorectal cancer cells it has been shown that the activation of AKT pathway and inhibition of the GSK-3β-dependent ubiquitination are required to induce Snail stabilization upon TNF-α treatment (Wang et al. 2013). In human prostate and lung cancer cells, EGF induces EMT by promoting Snail stability in a process mediated by protein kinase C and the inhibition of GSK-3β activity (Liu et al. 2014). The Wnt pathway promotes an Axin2-dependent stabilization of Snail by acting as a nucleocytoplasmic chaperone for GSK-3β (Yook et al. 2006). Data from a human kinome RNA interference screen pointed that Snail is also regulated post-transcriptionally by Lats2 kinase, that directly phosphorylates Snail in response to multiple signals, including TGF-β (Zhang et al. 2012). Other microenvironmental cues such as hypoxia or matrix modulators (Imai et al. 2003; Radisky et al. 2005) can induce Snail. For example, MMPs through ROS can stimulate the expression of Snail (Radisky et al. 2005).

Given the prominent role of Snail in inducing mesenchymal characteristics, it should be kept at a downregulated state in epithelial cells of adult tissues in order to maintain tissue homeostasis. In fact, during epithelial differentiation occurring in renal development, Snail is downregulated and its re-expression was shown to lead to kidney fibrosis (Boutet et al. 2006).

Slug

Slug (or Snai2) is member of the Snail superfamily. Some authors suggest that a duplication event originated *Snail* and *Slug* genes (Sefton, Sanchez, and Nieto 1998). Slug was first isolated in chick embryos, where it is expressed in neural crest cells delaminating from the neural tube (Nieto et al. 1994). Its role in EMT was proposed upon its silencing which results in improper mesoderm formation related to defects in cell migration (Nieto et al. 1994). Besides, *in vitro* overexpression of Slug induced changes in morphology, loss of desmosomal cell-cell adhesion structures, looser cell contact phenotype while its silencing inhibits EMT induced by FGF or HGF (Savagner, Yamada, and Thiery 1997). In epithelial kidney cells, Slug overexpression also induces highly migratory behavior and slower proliferation rate (Bolós et al. 2003). Knock-out mice for Slug, although viable, exhibit post-natal growth deficiency (Jiang et al. 1998).

Slug induces shared and specific gene expression patterns, when compared to Snail, upon their overexpression in epithelial cells (Moreno-Bueno et al. 2006). Similarly to Snail, Slug binds and represses E-cadherin expression (Bolós et al. 2003; Hajra, Chen, and Fearon 2002). The expression of other proteins is also directly affected by Slug, such as P-cadherin, that is preferentially expressed in the chick embryo epiblast, instead of E-cadherin, and similarly to E-cadherin in the mouse, is downregulated in the mesoderm and in the neural plate (Acloque et al. 2017). Slug has a key role in determining mammary cell identity, in a physiological or pathological setting, since its expression - together with Sox9 - is able to reprogram normal differentiated epithelial cells or non-metastatic cancer cells into stem cells or cancer cells with tumor-initiating and metastatic abilities, respectively (Guo et al. 2012).

Slug is essential for EMT in the heart organogenesis and its expression can be induced by TGF-β2 (Romano and Runyan 2000). Slug expression is also induced by FGF (Savagner, 1997), Notch (Leong et al. 2007), β-catenin, ERK (Conacci-Sorrell et al. 2003) and Wnt (Wu et al. 2012). So, Slug upregulation is mainly driven by signaling pathways commonly dysregulated in cancer, rather than genetic alterations on the gene itself, although, in humans, deletions of *SLUG* have been associated with Waardenberg syndrome (Sánchez-Martín et al. 2002). Moreover, several transcriptional factors and miRs can directly repress Slug expression (Zheng and Kang 2013). Slug protein stability is negatively regulated by GSK-3β and β-Trcp, which phosphorylate and ubiquitinate Slug, in a process regulated by Wnt signaling (Wu et al. 2012).

The differential expression of Slug has been observed in several types of cancer such as esophageal squamous cell carcinoma (Uchikado et al. 2005). Slug is associated with aggressiveness of the tumors (Alves et al. 2009), for example in breast cancer (Chakrabarti et al. 2012; Guo et al. 2012) and its overexpression leads to the development of mesenchymal cancers in mice (Pérez-Mancera et al. 2005). However, Slug expression is not always associated with E-

cadherin lost (Alves et al. 2009). Slug role in carcinogenesis is also evident in skin carcinoma where it collaborates with Snail in promoting metastasis (Olmeda et al. 2008).

Twist family

Twist belongs to the helix-loop-helix transcription factor family that includes Twist1 (that will be referred as Twist) and Twist2 (Twist-related protein), which are encoded by two genes in vertebrates (Barnes and Firulli 2009). Twist is able to induce EMT (Yang et al. 2004) and, like Snail, it promotes the cadherin switching, because it not only represses E-cadherin (Vesuna et al. 2008; Yang et al. 2004), acting in cooperation with the chromatin modifier Bmi1 (Yang et al. 2010), but also activates N-cadherin (Alexander et al. 2006; Oda, Tsukita, and Takeichi 1998). Like the previously mentioned EMT TFs, Twist was first studied in the context of embryogenesis and was shown to be essential for mesoderm formation (Simpson 1983), although the phenotype of the mutated *Drosophila* was different than the one expressing mutated *Snail* (Alberga et al. 1991). Mutations in *TWIST1* are associated with the autosomal dominant Saethre–Chotzen syndrome and these patients have a higher predisposition to develop breast cancer (Sahlin et al. 2007). Twist transcriptional role has, in fact, been highjacked by cancer cells and its expression seems to be required for intravasation and metastasis. Specifically, Twist is frequently overexpressed in human cancer (Ansieau et al. 2008) and Twist silencing has been shown to suppress the capacity of breast cancer cells to form lung metastasis (Yang et al. 2004).

In mammary epithelial cells, Twist expression is upregulated in response to Wnt1 (Howe et al. 2003), whereas in the cancer context, Twist expression can be triggered by TGF-β and the HRas pathway (Hong et al. 2011). Moreover, TNFα, through the NF-κB pathway, has been shown to upregulate Twist and conversely, Twist can regulate cytokine signaling thought a negative feedback loop that involves p65 (Šošić et al. 2003). MAPK pathway effectors p38, JNK and ERK1/2 can phosphorylate Twist, in this way stabilizing the protein by preventing its ubiquitination and consequent degradation (Hong et al. 2011). Not only cytokines and growth factors but also other microenvironment signals, such as oxygen availability, can regulate Twist expression. In fact, *Twist* promoter contains a functional HRE, which is the consensus DNA sequence for the binding of HIF; therefore, hypoxia can induce EMT through the binding of HIF-1α in the *Twist* promoter (Yang et al. 2008). Indeed, Twist was shown to be essential for hypoxia-induced EMT in hypopharyngeal cancer and breast cancer cell lines, despite the fact that Snail or lysyl oxidase are responsible for part of the migration and invasion activity not regulated by Twist (Yang et al. 2008).

Twist also protects cells from Myc-dependent apoptosis, mediates survival after DNA damage-induced stress, inhibits oncogene-induced senescence in cancer cells and was shown to be

important for tumor cell proliferation (Ansieau et al. 2008; Maestro et al. 1999; Qian et al. 2013; Shiota et al. 2008; Vichalkovski et al. 2010), although that is not always the case (Yang et al. 2004).

Zeb family

Zeb1 and Zeb2 belong to the Zeb family of zinc finger/homeodomain transcription factors. In this thesis, I will use the name *ZEB1* to refer to the gene that has also been called *AREB6* (in humans), *BZP* (in hamster), or δ*EF1* (in chicken) (GenBank™ accession number U12170) and *ZEB2* for the gene also known as *SIP1* (GenBank™ accession number AB011141).

Zeb TFs downregulate E-cadherin by direct repressing the transcription of its gene (Comijn et al. 2001; Grooteclaes and Frisch 2000). Like *Snail* mutants, *Zeb2* mutant embryos maintain E-cadherin expression in the neural plate and the presumptive neural crest, causing several neural crest defects and die after neurulation (Van de Putte et al. 2003). In cancer cells, Zeb2 expression induces changes in morphology, invasion and loss of aggregation, together with alterations in the expression of several genes that encode adhesion proteins (Vandewalle et al. 2005). As an example, Zeb2 was shown to induce P-cadherin expression in the context of chick embryonic development and human cancer (Acloque et al. 2017; Vandewalle et al. 2005). Zeb proteins are part of the CtBP complex that recruits histone methyltransferases among others, being CtBP involved in repression of the *CDH1* promoter (Furusawa et al. 1999; Postigo and Dean 1999, 2000; Shi et al. 2003). Besides, the transcriptional repression by Zeb1 can also be mediated through recruitment of other histone modifiers, such as the SWI/SNF chromatin remodeling ATPase BRG1 (Sánchez-Tilló et al. 2010). One of the most well studied regulators of Zeb expression is the miR200 family, which has been considered a marker for epithelial cells and a regulator of EMT (Korpal et al. 2008; Park et al. 2008).

The expression of Zeb proteins is activated by several signaling pathways like TGF-β (Comijn et al. 2001), HRas (Barberà et al. 2004; Shin and Dimitri et al. 2010) and Wnt (Sánchez-Tilló et al. 2011). Zeb1/2 was found to mediate the transition to a mesenchymal phenotype driven by ERK2 DEF motif signaling, downstream of Fra1 (Shin, Dimitri et al. 2010). Interestingly, Snail itself induces the expression of Zeb1 in cancer and non-transformed epithelial cells, through an increase in Zeb2 promoter activity (Guaita et al. 2002).

Some of the main EMT inducers and effectors, as well as the described alterations in cellular phenotype are summarized on fig. 10.

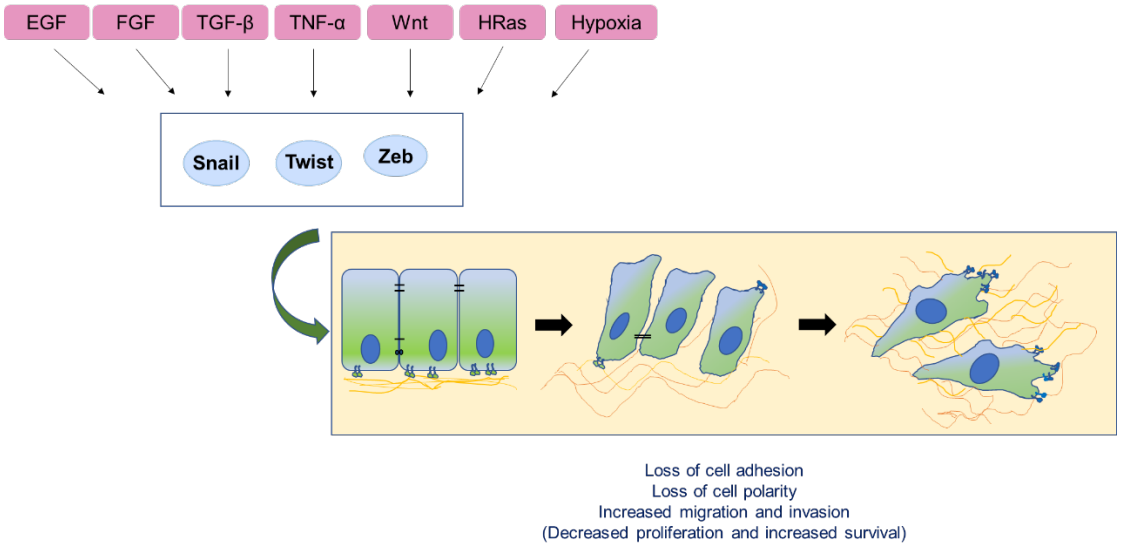

Fig. 10: Epithelial to mesenchymal transition (EMT): some inducers, major EMT transcription factors and phenotypic alterations.

1.3.3 - When does it occur?

EMT mainly occurs in 3 different settings: during embryonic development, during the wound healing response and fibrosis and in cancer (Fig. 11). The EMT process was firstly studied and it is well-characterized in the developmental stages of the embryo and it was later expanded to other settings.

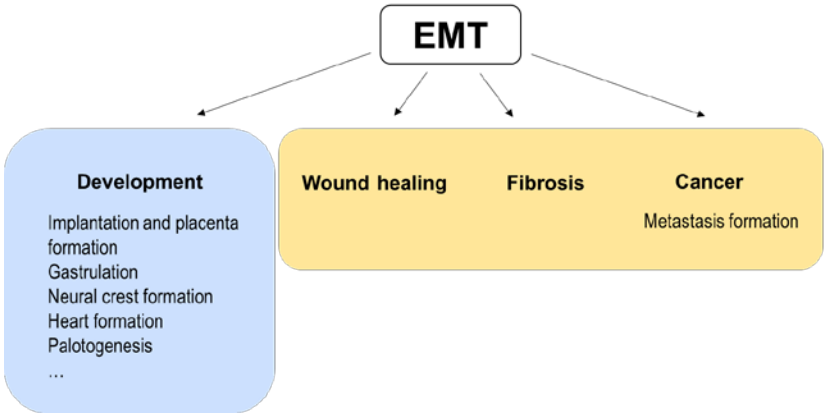

Fig. 11: Processes where epithelial to mesenchymal transition has been described.

Elizabeth Hay was one of the researchers who had a strong impact on the study of the “epithelial to mesenchymal transformation”, as it was first called. It was known that in the embryo (studies on chick embryo development), epithelial cells give rise to mesenchymal cells (Trelstad, Hay, and Revel 1967); however, the epithelial phenotype of cells was considered very stable. It was

then observed that epithelial cells from tissues explants, that normally do not form mesenchyme, when cultured in a gelatin solution of collagen, spread apart, loose their polarity and migrate (Greenburg and Hay 1982). Particularly, the heart development was one of the first *in vitro* experimental systems where EMT was studied. The detachment and invasion of myocardium by endothelial cells of the atrioventricular canal was studied in the 80s focusing on the role of extracellular matrix, having suggested the presence of factors that induce changes in endothelial cells (Krug, Mjaatvedt, and Markwald 1987; Runyan and Markwald 1983). Hay early studies also concern the mechanism for palate fusion, inspired by findings in the rat reproductive organs where EMT plays a role in the remodeling of embryonic tissues (Trelstad et al. 1982) and not only in the early embryo. In fact, they first showed that epithelial cells from the medial edge become elongated, form filipodia and pseudopodia, lose keratin expression and start expressing vimentin suggesting that epithelial to mesenchymal transformation underlies the mechanism of the disappearance of the midline epithelial stem (Fitchett and Hay 1989).

EMT in embryonic development

EMT is a key developmental process. It happens during numerous stages of development and it is a reversible process. EMT is essential for the formation of the embryo in several stages such as before implantation, during gastrulation and neural crest formation. Moreover, the differentiation events that give rise to the heart and the palate were some of the first models where EMT was studied.

Epithelia is the first tissue to be formed. For example, in amniote vertebrates, the embryo derives from an epithelial layer of cells called the epiblast. Then, a second tissue composed of cells with the ability to migrate appears as a result of EMT. Interestingly, amphioxus, a chordate ancestor, evolutionarily diverged before skeletal tissue was originated and only has epithelial tissue, suggesting that ontology repeats phylogeny. The appearance of the mesenchyme reflects the generation of motile cells, which are able to move into an extracellular matrix that provides organs with shape and strength, supporting the emergence of bigger structures (Hay 2005).

<u>Implantation and placenta formation</u>

During early embryogenesis, the cells from the outer trophectoderm, after blastocyst migration to the uterine cavity, adhere to the uterine wall. These cells undergo EMT enabling them to migrate and invade the endometrium, which facilitates the development of connections with the maternal blood supply and formation of the placenta (Kokkinos et al. 2010; Sutherland 2003). E-cadherin,

a key molecule in EMT regulation plays an important role in both the induction and orientation of polarity (Kokkinos et al. 2010).

Gastrulation

Gastrulation occurs after blastocyst formation and is the developmental stage when the body axes are defined and the three germ layers are produced. This occurs in result of the formation of a region where cells involute or ingress and of cell movements. In amniote vertebrates, the primitive streak forms upon the convergence of epiblast cells at the midline of the epiblast layer. The migration (ingression) of mesendodermal precursors through the primitive streak, which generates the mesoderm, involves EMT. Then, these mesenchymal cells migrate from the primitive streak, occupy different positions along the medio-lateral axis of the embryo and differentiate into the mesodermal and endodermal epithelia, presumably through mesenchymal to epithelial transition (MET) (Ferrer-vaquer, Viotti, and Hadjantonakis 2010).

A second round of EMT, undergone by the epithelial mesoderm, is necessary to form the definitive mesenchyme that differentiates into specific cell types, such as the vertebras, hematopoietic cells, connective tissue of the muscle, palate, pancreatic endocrine cells and cells from the reproductive tract (Thiery et al. 2009).

From invertebrates, like the sea urchin and flies, to *Xenopus* and mice, there is a sharing of various players involved in gastrulation (Thiery et al. 2009). Focusing on mammals, E-cadherin is expressed in the cells of the epiblast and then it becomes downregulated in the migrating mesoderm cells, where Snail is expressed (Damjanov, Damjanov, and Damsky 1986; Nieto et al. 1992). Mice deficient in Snail die before birth, exhibiting defects in gastrulation and particularly, failing to downregulate E-cadherin (Carver et al. 2001).

FGF is a key regulator of EMT in mammalian gastrulation. Using primitive streak explants and transgenic mice embryos, it was shown that FGF receptor 1 signaling is required for maintenance of Snail expression in the late primitive streak and for E-cadherin loss. Importantly, FGF signaling also regulates Wnt and β-catenin, two important developmental proteins (Ciruna and Rossant 2001). On the other hand, FGF also plays a role in mesoderm cell fate determination (Ciruna and Rossant 2001).

Besides being regulated at the transcription level, E-cadherin protein levels are also regulated during gastrulation. For example, p38-interacting protein, which binds and activates p38, is required for a proper downregulation of E-cadherin during mouse gastrulation, in a FGF receptor 1/Snail independent way (Zohn et al. 2006).

Neural crest formation

The ectoderm forms from the epiblast during gastrulation and it is the external germ layer in the embryo. It originates the nervous system and the epidermis. The neural crest is a group of stem/progenitor cells that appears after neurulation in between the surface ectoderm and the neuroepithelium of the central nervous system. In contrast to the epidermis, the emergence and migration of neural crest cells from the dorsal neural tube involves EMT. Neural crest cells originate cells of the peripheral nervous system, bone, cartilage, melanocytes, and also tissues of non-neural crest origin, like bone marrow, spleen or cardiac muscle (Zurkirchen and Sommer 2017).

During neural crest formation, a complex network of cooperating pathways is responsible for the regulation of cell polarity, adhesion and migration (Thiery et al. 2009). The differential expression of cadherins or "cadherin switching" is one of the key features in the formation of the neural crest. For example, cadherin 6B is required for EMT and mediates de-epithelization through BMP signaling (Park and Gumbiner 2010), which simultaneously inhibits N-cadherin expression in the dorsal neural tube (Shoval, Ludwig, and Kalcheim 2007). The Wnt pathway, specifically the non-canonical arm, also plays an important role in neural crest migration (De Calisto et al. 2005; Dorsky, Moon, and Raible 1998).

Heart formation

The heart is the first functioning organ in the embryo and it that arises from multiple (three) rounds of EMT/MET. The first round occurs during gastrulation and originates the cardiac mesoderm. A secondary EMT happens when endocardial progenitors are formed. Finally, the atrioventricular canal and the outflow tract generate the cushion mesenchyme, which is the precursor of the cardiac valves, through a third round of EMT (Person, Klewer, and Runyan 2005). Some authors suggest that this third transition should be designated as endothelial to mesenchymal transition (Nieto et al. 2016). Several pathways contribute to the morphogenetic program of EMT, particularly TGF-β, BMPs, Notch and ErbB (Brown et al. 1999; Camenisch, Molin, et al. 2002; Camenisch, Schroeder, et al. 2002; Luna-Zurita et al. 2010).

Palatogenesis

During palatal seam disintegration, the final stage of palate development, epithelial cells from the palate medial edges are replaced by mesenchymal cells. It has been proposed that this process, which happens during palate fusion, one of the critical phases of during palate development,

occurs through programmed cell death or EMT or both (Nawshad 2008). The two palate halves grow out from the internal surfaces of the maxillary processes and then elongate and ascend to a horizontal position above the tongue where they fuse, forming a medial epithelial seam. When a single layer of epithelial cells is created, the palatal epithelial seams disintegrates, a phenomenon mediated by TGF-β3, Smad4, Snail and Zeb2 signaling. E-cadherin promoter activity was shown to be repressed after TGF-β3 treatment, through Smad4 (activated by the Smad-dependent pathway), Snail and Zeb2 (activated by Smad-independent pathways) interaction with the *CDH1* promoter (Jalali et al. 2012). TGF-β3 signaling is also essential for other steps of palate fusion such as the filopodia formation by epithelial cells prior the contact of the opposing palatal shelves (Taya, O'Kane, and Ferguson 1999).

The study of the role of EMT in palate formation can be of extreme value in a therapeutic perspective, since cleft palate (without cleft lip) is a birth defect affecting 6.35/ 10,000 live births in the United States of America (Parker et al. 2010).

EMT in wound healing and fibrosis

From physiology to disease setting, EMT is important during the process of tissue repair upon injury to the epithelium and during tissue fibrosis. These processes share several cellular and molecular features with EMT. Indeed, growth factors that have been linked to EMT induction *in vitro* are major inflammatory mediators, which are released after injury: TNF-α, TGF-β, nuclear factor kappa B subunit 1 (NF-κB), several interleukins are some examples (Suarez-Carmona et al. 2017).

Wound healing

Upon injury to the epithelial tissue, the restoration of the epithelial layer involves the proliferation and migration of epithelial cells surrounding the wound. In the adult skin, after injury, clot formation and vasoconstriction to control the bleeding are followed by the inflammatory process, in which immune cells infiltrate the wound. Then, the proliferation stage starts, being is characterized by re-epithelization, angiogenesis, collagen synthesis and extracellular matrix formation. Finally, the tissue remodeling can last for years (Guo and DiPietro 2010). In the wound bed, during the proliferation stage, the stratified epithelium has to be regenerated to seal the wound; in this process, keratinocytes, which are the main constituent of the epidermis, have to undergo changes in their morphology and function. These cells migrate from the edges of the wound and this is accompanied by proliferation, stratification and redifferentiation to form an intact epithelium (Arnoux et al. 2005).

It was first proposed by Arnoux *et al.* that the alterations experienced by keratinocytes during re-epithelization are reminiscent of EMT (Arnoux et al. 2005). Keratinocytes start expressing different keratins and integrins (Larjava et al. 1993; Paladini et al. 1996), which are molecular events described in other types of EMT. Adjacent to the wound, epithelial cells are arranged into two spatially different zones: a proliferative hub and a migrating leading edge. Migration and proliferation seem to be fairly mutually exclusive as the cells in the migratory front are not actively proliferating (Aragona et al. 2017), which is consistent with EMT. Epithelial cells show re-localization of E-cadherin (Arnoux et al. 2005) and its downregulation has been observed in the basal layer (Kuwahara et al. 2001). Besides, epithelial cells express mesenchymal markers, such as fibroblast-specific protein-1 (FSP-1) and/or vimentin in acute and fibrotic wounds in human skin and TNF-α induces EMT in cultured primary human keratinocytes (Yan et al. 2010).

Slug is a transcription factor that modulates wound re-epithelization. This molecule is expressed by migrating keratinocytes in the wound edge and is necessary for their outgrowth in skin explants (Savagner et al. 2005). It was also demonstrated that mice lacking Slug show a slower re-epithelization in excisional wounds and develop non-healing cutaneous ulcers triggered by chronic exposure to ultraviolet radiation (Hudson et al. 2009). Moreover, EGFR through ERK5 upregulates Slug expression in immortalized keratinocytes which is essential for EGF-induced re-epithelization (Valerie Arnoux et al. 2008).

In the final stages of re-epithelialization, keratinocytes regain the epithelial phenotype, with the formation of stable intercellular and cell–substrate contacts (desmosome reassembly). For example, after the meeting of the epithelial tongue, when migration stops, E-cadherin expression is recovered (Kuwahara et al. 2001).

Fibrosis

I will mainly discuss fibrosis of the lung, liver and kidney, where the EMT hypothesis has been more extensively studied. However, since these tissues present distinct tissue architectures, their fibrosis program may show unique features. Fibrosis occurs during several chronic diseases and ultimately can lead to organ failure and destruction, being a major cause for morbidity and mortality. Fibrosis has also been shown to be important for tumorigenesis.

Fibrosis is characterized by the excessive deposition of connective tissue and is the pathological end point in the response to inflammation. The myofibroblasts or activated fibroblasts that appear after tissue injury are responsible for the production of extracellular matrix. These cells are usually identified by the *de novo* expression of α-smooth muscle actin (α-SMA) (Hinz et al. 2007), although this is not universal (Okada et al. 2000). Depending on the tissue, myofibroblasts can

have several origins: they can be derived from quiescent fibroblasts or derived from other cell types of mesenchymal origin that reside in the tissue or that come from circulating bone-marrow-derived progenitor cells. It has also been suggested that, in certain organs, activated fibroblasts can originate from the transformation of endothelial or epithelial cells, which undergo endothelial to mesenchymal transition or EMT, respectively. Concerning EMT, after the characterization of FSP-1 as a marker for fibroblasts allowing the analysis of their distribution in the fibrotic tissue and the study of the effects of FSP-1 overexpression in epithelial cells, it was hypothesized that fibroblasts in some cases arise, as needed, from the local conversion of epithelium (Strutz et al. 1995).

Indeed, part of the evidence that supports the role of EMT in fibrosis comes from the histopatological and immunohistochemistry analyses that revealed the presence of mesenchymal markers in fibrotic tissues (Díaz et al. 2008; Rastaldi et al. 2002; Xu-Dubois et al. 2014). Yamaguchi *et al*. proposed that fibroblastic foci in idiopathic pulmonary fibrosis, which are a hallmark of the disease, are derived from EMT since they are surrounded by alveolar epithelial cells and the latter express both epithelial and mesenchymal markers (Yamaguchi et al. 2017).

TGF-β1 is thought to be a major regulator of fibrosis progression in several tissues. Strategies that target the pro-fibrotic roles of this pathways have been highly studied in the last years, with a few approaches in clinical trials (reviewed in Walton, Johnson, and Harrison 2017). *In vitro* studies showed that alveolar epithelial cells, hepatocytes and renal tubular epithelial cells undergo EMT upon TGF-β treatment (Humphreys et al. 2010; Nitta et al. 2008; Rastaldi et al. 2002; Willis et al. 2005; Yamaguchi et al. 2017). In the particular case of liver and lung, MAPK pathway seems to be an important mediator of this process (Godoy et al. 2009; Huang et al. 2016; Li et al. 2017; Nitta et al. 2008).

In vivo tracing studies emerged with the hope of shedding some light into the contribution of EMT to the formation of myofibroblatsts, however different studies have come to different conclusions. In the liver and upon inducing fibrosis with carbon tetrachloride, FSP1 positive fibroblasts contribute to the progression of liver fibrosis and can be derived from hepatocytes *in vivo* (Michael Zeisberg et al. 2007). On the other hand, a similar study showed that hepatocytes do not originate cells positive for type-I collagen nor do they acquire a mesenchymal morphology (Taura et al. 2010). The contribution of EMT for the formation of myofibroblasts present during fibrosis has also been assessed in the lung using genetic manipulation of mice. Tanjore et al. showed that a small subset of fibroblasts is originated from lung epithelium through EMT in bleomycin-induced lung fibrosis (Tanjore et al. 2009). However, a study using a different lineage labeling system showed that epithelial cells originated from two progenitor populations do not generate myofibroblasts in the context of pulmonary fibrosis (Rock et al. 2011). In the kidney, Iwano *et*

al. has also shown that a percentage of cells expressing mesenchymal markers can have epithelial origin (Iwano et al. 2002), a study that was subsequently supported in more detail by other authors (LeBleu et al. 2013). Nevertheless, there is also evidence that pericytes, and not epithelial cells, are the main source of myofibroblasts after injuries that lead to interstitial fibrosis (Humphreys et al. 2010).

As such, it is still debatable if EMT is indeed a contributor for the formation of activated fibroblasts in fibrosis of several organs (Kriz, Kaissling, and Le Hir 2011; Munker et al. 2017; Zeisberg and Duffield 2010). One of the most controversial questions is the necessity, or not, of epithelial cells acquiring a migratory potential in order to lead to fibrosis. This is one of the points where tissue architecture may be an important factor and may explain the weight of the EMT hypothesis in fibrosis of different tissues.

Interestingly, the knock-out of EMT transcription factors revealed that EMT may play a more complex role in fibrosis. Snail, a transcription factor that induces the EMT program (as discussed previously) seems have an important role for the transition of renal cells from mesenchymal to epithelial during development, by regulating cadherin-16 through hepatocyte nuclear factor-1β (HNF-1β) and its reactivation is sufficient to induce fibrosis *in vivo* (Boutet et al. 2006). A study using transgenic mice where the expression of Snail could be traced *in vivo* and subjected to unilateral ureteral obstruction showed that Snail is actually required for the development of renal fibrosis by triggering a partial EMT in epithelial cells. However, these epithelial cells maintain the integrity of the tubular tissue and do not contribute directly for the myofibroblast pool but rather promote it by secreting of signals like TGF-β (Grande et al. 2015). This partial EMT is supported by a back-to-back study that also added that the EMT program is associated with a G2 cell cycle arrest and with alterations in transporter proteins in tubular epithelial cells which require Snail and Twist (Lovisa et al. 2015).

The non-concordant results about the contribution of EMT to the generation of activated fibroblasts can be explained by the stringency of the definition of EMT (complete EMT) and the possibility of partial EMT. However, in this case, if a “completely mesenchymal” fibroblast phenotype is not achieved, the functionality of a partial EMT is still lacking, some authors argue (Kriz, Kaissling, and Le Hir 2011).

Many papers have shown that epithelial cells change their morphology and undergo dedifferentiation after injury but it has been very disputed if those changes can be termed EMT. The models used are heterogeneous and use a limited number of markers for *in vivo* tracing. One of the problems with *in vivo* tracing studies is the efficiency and specificity of the Cre expression system. The fibroblast markers used are also still debatable (α-SMA versus FSP-1) (Kriz, Kaissling, and Le Hir 2011).

EMT and cancer

Carcinomas arise from epithelial cells, the latter being characterized by strong cell-to-cell contacts that limit their ability to move and invade, which are eventually lost during cancer progression. The EMT program can be reactivated during the metastasis process of carcinomas, especially in one of the first steps towards the colonization of the secondary tumor site, when tumor cells detach from the primary tumor, migrate and invade the adjacent tissue and intravasate into the vascular system. EMT confers tumor cells the ability to migrate and invade, having been proposed to be one of the cell-biological programs that enables dissemination (Thiery et al. 2009).

Several lines of evidence implicate EMT in tumor progression. In 1989, Boyer *et al.* reported that epithelial bladder carcinoma cells cultured in matrixes undergo phenotypic changes characteristic of EMT, namely cellular morphology, adhesion and motility (Boyer et al. 1989). The presence of mesenchymal traits has been reported in several types of epithelial-derived tumors (Elloul et al. 2005; Uchikado et al. 2005; Vasko et al. 2007; Yang et al. 2004). Particularly, the expression of mesenchymal-associated proteins is seen in cells located at the leading edge of the tumor (Brabletz et al. 2001; Prall 2007; Vasko et al. 2007). In transgenic *in vivo* models of mammary tumors, the expression of EMT makers linked to a reporter gene allowed the identification of Snail expressing cancer cells in the tumor, being these cells detached from the epithelial islands and exhibiting an elongated mesenchymal morphology (Ye et al. 2015). In fact, one of the first studies using transgenic mice showing the importance of EMT for metastasis was performed by Yang *et al*., who observed that Twist inhibition suppressed metastasis formation (Yang et al. 2004). The development of fate tracing techniques was crucial in defining the presence of EMT in cancer cells *in vivo* (Tran et al. 2014; Trimboli et al. 2008). Additionally, the presence of EMT markers is associated with metastasis and poor prognosis in esophageal squamous cell carcinoma, ovarian, breast cancer, gastric cancer, among others (Elloul et al. 2005; Ryu et al. 2012; Uchikado et al. 2005). For example, after analyzing the expression of EMT markers in 1495 breast cancer biopsies, it was found that higher E-cadherin expression and lower expression of fibronectin is associated with longer survival (Bae et al. 2015). E-cadherin was one of the first proteins to be implicated in EMT during tumorigenesis, being associated with cell invasion and tumor progression (Frixen et al. 1991; Perl et al. 1998). Cadherins are, indeed, a family of adhesion proteins that have a primordial role in carcinoma progression (van Roy 2014). For example, the co-expression of E-cadherin and P-cadherin is associated with poor overall survival in breast carcinoma (Paredes et al. 2008).

The tumor microenvironment has a primordial role in inducing EMT. Therefore, EMT is probably a focal rather a general event (Nieto et al. 2016). For example, mesenchymal cells generated spontaneously *in vitro* from immortalized human mammary epithelial cells actually arise and are

maintained by several factors that function in a paracrine and autocrine manner (Scheel et al. 2011). Cells present in the tumor stroma, like macrophages, cancer-associated fibroblasts and adipocytes can produce signals that induce EMT (Bottai et al. 2016; Lee, Jung, and Koo 2015; Rupp et al. 2014; Vellinga et al. 2016; Wyckoff et al. 2004; Yu et al. 2013).

When does EMT occur during tumorigenesis? The presence of EMT in pre-neoplastic lesions of breast and pancreatic cancer suggests that EMT is an early event during tumorigenesis (Harper et al. 2016; Hüsemann et al. 2018; Rhim et al. 2012). In breast cancer, a population of cells from tissues of early lesions that can be identified even before the detection of primary tumor masses, activate an EMT-like response, being characterized by a high expression of Twist and low E-cadherin expression (Harper et al. 2016).

EMT and metastasis: MET and partial EMT

EMT was first described as an "epithelial to mesenchymal transformation"; however, with the discovery that EMT is a reversible process in development this denomination was changed into "epithelial to mesenchymal transition" to better reflect cell plasticity (Nieto et al. 2016).

The fact that the cancer cells from the metastasis resemble histopathologically the ones from the primary tumor, in many cases presenting a well-differentiated, epithelial morphology, and not the migratory cells that are thought to be the ones initiating the metastasis process was an apparent paradox (Ng 2002; Rubin et al. 2000; Tan et al. 1999). Besides, the presence of cells expressing epithelial proteins in invasive or metastatic lesions suggested a lack of a complete transition or a reversion of the phenotype (Hashizume et al. 1996; Kartenbeck, Haselmann, and Gassler 2005; Mareel et al. 1991). For example, metastases of breast cancer are typically epithelial (Kowalski et al., 2003).

Today we can explain these observations in several ways. One is that EMT is just a mechanism, among many others, associated with cancer cell dissemination and metastatic outgrowth (Lambert, Pattabiraman, and Weinberg 2017). On the other hand, a complete transition to a mesenchymal phenotype in not always required for tumor cell invasion. Perhaps most important is the finding that when circulating tumor cells arrive at their destination, they generally undergo the reverse process of EMT, denominated MET.

In a study performed in an *in vivo* breast cancer model where mammary epithelial cancer cells were orthotopically implanted in the mammary fat pads of mice, which analyzed the expression of EMT markers in the cells from the primary tumor, the cancer cells in circulation and the cells from the metastasis showed that circulating tumor cells upregulate Twist, Snail and αSMA compared to the primary cancer cells. Moreover, the expression of these EMT-associated genes

was shown to be reversible since the cancer cells from the metastasis and the ones from primary tumor show similar levels of expression (LeBleu et al. 2014).

Numerous *in vivo* experimental systems have shown that MET is required for actively proliferating metastases (Chaffer et al. 2006; Ocaña et al. 2012; Tsai et al. 2012). Ocaña *et al.* demonstrated that Prrx1 (paired-related homeobox transcription factor 1), an EMT inducer in embryos and cancer cells that cooperates with Twist, must be downregulated for metastasis to occur (Ocaña et al. 2012). Using an inducible reporter system *in vivo*, Tran *et al.* showed that Snail is expressed in primary breast tumors but then lost in tumor cells that disseminated to the bone-marrow and in lung metastases (Tran et al. 2014). The downregulation of Twist is also essential for the establishment of macrometastases (Tsai et al. 2012). Moreover, it was also shown in a *in vivo* breast cancer model that forced and prolonged activation of TGF-β signaling impairs the formation of lung metastasis showing the importance of a cell being able to downregulate TGF-β signaling at certain phases of tumor progression (Giampieri et al. 2009). A prolonged EMT signal may indeed be detrimental for the cells. A transient Twist activation in human mammary epithelial cells, rather than constitutive, primes a subset of cells for stem-cell-like traits that emerge as stable traits after Twist1 deactivation (Schmidt et al. 2015). Interestingly, this short-term Twist activation permanently alters cell state (Schmidt et al. 2015). Interestingly, some evidence points to the fact that when cells undergo MET, they do not return to their original epithelial cell state, rather remaining in an intermediate state (Schmidt et al. 2015).

Another interesting concept that may unify the field of EMT is that EMT reflects cell plasticity. Some authors propose that a complete transition to a mesenchymal phenotype is not required for invasion and metastasis and a graded range of intermediate states may exist (Christiansen and Rajasekaran 2006; Kalluri and Weinberg 2009; Nieto 2013).

In some of the cases where EMT occurs, a “complete” mesenchymal end point is not observed but rather a mix of epithelial and mesenchymal markers. This has been reported not only in the context of cancer but also wound healing and fibrosis (Valerie Arnoux et al. 2008; Harper et al. 2016; Pinkas and Leder 2002; Yamaguchi et al. 2017). Cells from early lesions originated in a breast cancer transgenic model, besides EMT features, still expressed cytokeratins 8 and 18, suggesting that a partial EMT is sufficient for early dissemination, dormancy and metastasis formation (Harper et al. 2016). Another aspect that supports the existence of a partial EMT in cancer cells is the presence of epithelial and mesenchymal markers in circulating tumor cells (Yu et al. 2013). This also reinforces the active role of EMT for dissemination of cancer cells from the primary tumor site and intravasation. The retention of epithelial features may bring advantages during dissemination, since clusters of tumor cells in circulation exhibit increased metastatic

propensity compared to single cells (Aceto et al. 2014). Besides, partial EMT may also be advantageous for collective migration of cancer cells during the delamination form the primary tumor (Campbell and Casanova 2016).

In 2015, two back-to-back studies analyzed the contribution of EMT to metastasis formation, bringing a new insight on the role of EMT in chemoresistance. Fischer *et al.*, built an *in vivo* system where the reporter activation caused by expression of the mesenchymal markers Fsp-1 and vimentin is irreversible, allowing to monitor EMT by color change, even if cells reversed. They showed that primary breast tumors and derived lung metastasis of transgenic or orthotopic mouse models are mainly composed of cells that did not activate the expression of Fsp-1 or vimentin (Fischer et al. 2015). It was also shown that the number of cells in the metastasis that had undergone EMT increased after the mice were treated with cyclophosphamide, a chemotherapeutic agent, indicating these cells are more resistant to chemotherapy (Fischer et al. 2015). Similarly, Zheng *et al.* showed that loss of Snail or Twist does not block metastasis formation in a mice PDAC model, but increases chemotherapy sensitivity (Zheng et al. 2015). The authors of both studies concluded that EMT does not significantly contribute to the development of lung metastasis. However, different interpretations have been suggested by others, who also have pointed some limitations of these studies, especially concerning the markers used to trace EMT (Nieto et al. 2016).

In fact, pathways that regulate EMT are not universal and we still need to further understand which EMT effectors are activated in the different cellular contexts. For example, in breast tumors, the expression of Snail and Zeb1 rather than Slug is associated with the invasive cells (Ye et al. 2015).

Importantly, some cancers may metastasize without the need for an EMT program, as EMT is not the only phenomenon that has been associated with metastasis. Cancer cells can invade and enter the vascular system through collective epithelial-cell migration (Giampieri et al. 2009). For example, the formation of heterodimers composed of E-cadherin and N-cadherin between cancer-associated fibroblasts and cancer cells can mediate the cancer cell invasion (Labernadie et al. 2017).

Concluding, although not being a guiding principle for all cancers, evidence supports an important contribution of EMT for the formation of metastasis especially in the first steps of the metastatic cascade in carcinomas.

EMT and stemness

The role of EMT in the acquisition of stem cell properties has become a research area of particular interest. Some authors propose that certain epithelial cells that undergo EMT acquire the self-renewing trait associated with normal tissue stem cells and cancer stem cells (Polyak and Weinberg 2009). For example, EMT induced by the overexpression of Twist or Snail or activation of the Ras/MAPK signaling pathway is associated with a $CD44^{high}/CD24^{low}$ expression pattern that characterizes mammary tumor stem cells and the capability of forming mammospheres, which are stem cell-enriched structures (Mani et al. 2008; Morel et al. 2008). In breast cancer, the $CD44^{high}/CD24^{low}$ cell population exhibits the expression of epithelial and mesenchymal proteins (Goel et al. 2014).

However, in some instances, stemness and EMT can be uncoupled. In a benign skin tumor model, low levels of Twist are required for tumor cell proliferation and tumor stemness through a yet unknown mechanism, whereas high levels of Twist1 switch cells into a migratory mode with reduced proliferation to promote metastasis (Beck et al. 2015). This suggests that it is not the EMT program *per se* that confers cancer stem cell properties, but these can be attributed to the pleiotropic functions of EMT TFs.

Targeting EMT in cancer

EMT in cancer is associated with progression and drug resistance, two of the greatest challenges in oncology. Therefore, EMT has gained increasing interest as a potential therapeutic target, especially to overcome cancer drug resistance (Fischer et al. 2015; Zheng et al. 2015). This complex process offers several targeting strategies, for example by blocking extracellular signaling molecules or EMT signal transduction pathways, such as EMT TFs. Some authors have suggested targeting the players in EMT, for example, the cadherin switching, which can be achieved by 5-aza-2′-deoxycytidine to restore normal *CDH1* promoter methylation patters and E-cadherin expression (Graff et al. 1995). Alternatively, in lung cancer inflammation-associated factors like TGF-β and interleukine-6 contribute to primary and acquired erlotinib (a receptor tyrosine kinase inhibitor that acts on EGF receptor) resistance (Yao et al. 2010). However, many of the molecules involved in EMT have important roles in physiological processes such as wound healing. Moreover, although in the recent years a significant number of studies examining the role of EMT in cancer has emerged, the interplay between different signaling pathways that drive EMT is more complex than we initially thought. Importantly, targeting cancer cells EMT may be a double-edged sword, since inhibiting EMT may promote the colonization of secondary tumor sites when they are already disseminated cancer cells. Despite these challenges, there may be potential benefits in tempering cancer EMT in combination with existing chemotherapeutic agents.

2 - GENERAL AIMS AND OBJECTIVES

The reprogramming of cellular metabolism is emerging as a key feature of cancer cells after the pioneer work of Otto Warburg, who showed that cancer cells display increased aerobic glycolysis. Warburg proposed that this metabolic phenotype was due to an impaired mitochondrial respiration, although the causes and the tumorigenic effects resulting from the "Warburg effect" are still unclear. Therefore, in the first part of this thesis, we set out to understand if a defective OXPHOS due to mtDNA mutations impacts cancer cell metabolism - consistent with the "Warburg effect"? - and what are the consequences on cancer cell behavior.

In the second part, we aimed at revealing the metabolic requirements of EMT, taking into consideration that EMT and the presence of mesenchymal markers in human tumors are associated with increased migration and invasive properties, the latter being key for cancer cell dissemination (Fig. 12). The relevance of this part of the thesis is to put forward new, metabolic-linked mechanisms that control EMT and to deliver metabolic players that are crucial for EMT pathways. These players may be candidate targets for cancer therapy, particularly for aggressive tumors that often display mesenchymal markers. This metabolic reprogramming can confer selective advantage for tumor progression and, therefore, be a source of vulnerability and therapeutic opportunity.

Below, we describe our specific objectives for each part of this work.

PART 1: Metabolic and oncogenic properties of OXPHOS-deficient cells associated with mtDNA alterations

1) Establish cybrid cell lines with defects in OXPHOS caused by a mtDNA mutation (Paper I);

2) Evaluate the metabolic changes induced by the OXPHOS dysfunction (Paper I);

3) Assess the consequences of OXPHOS dysfunction on the behavior of cancer cells *in vitro* and *in vivo* (Paper I);

PART 2: The metabolic requirements of cancer-associated EMT

1) Investigate the metabolic alterations associated with an *in vitro* ERK2-driven model of EMT (Paper II);

2) Determine to what extent EMT induction is dependent on such metabolic alterations (Paper II);

3) Characterize the networking between activated metabolic pathways and EMT signaling pathways (Paper II).

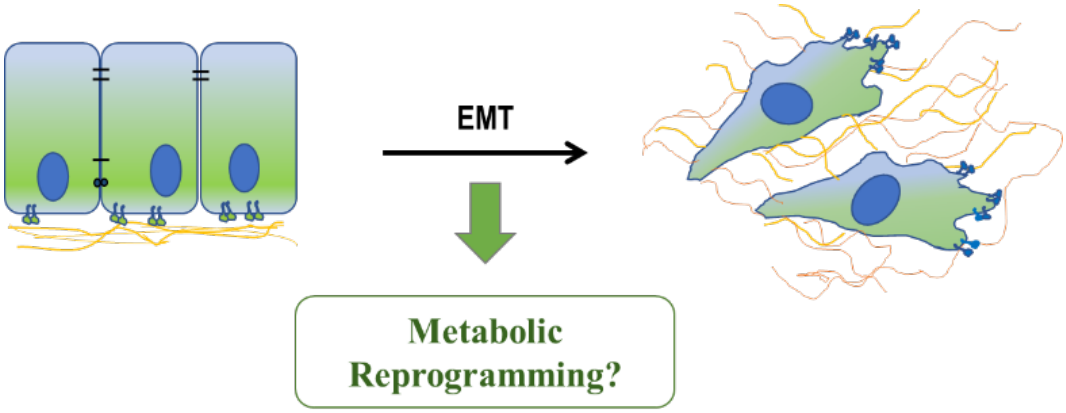

Fig. 12: Aim of part 2 of this thesis: to understand the metabolic requirements of cancer-associated EMT.

3 – RESULTS

3.1 – PAPER I: OXPHOS Dysfunction Regulates Integrin-β1 Modifications and Enhances Cell Motility and Migration

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ORIGINAL ARTICLE

OXPHOS dysfunction regulates integrin-β1 modifications and enhances cell motility and migration

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Abstract

Mitochondria are central organelles for cellular metabolism. In cancer cells, mitochondrial oxidative phosphorylation (OXPHOS) dysfunction has been shown to promote migration, invasion, metastization and apoptosis resistance. With the purpose of analysing the effects of OXPHOS dysfunction in cancer cells and the molecular players involved, we generated cybrid cell lines harbouring either wild-type (WT) or mutant mitochondrial DNA (mtDNA) [tRNAmut cybrids, which harbour the pathogenic A3243T mutation in the leucine transfer RNA gene (tRNAleu)]. tRNAmut cybrids exhibited lower oxygen consumption and higher glucose consumption and lactate production than WT cybrids. tRNAmut cybrids displayed increased motility and migration capacities, which were associated with altered integrin-β1 N-glycosylation, in particular with higher levels of β-1,6-*N*-acetylglucosamine (GlcNAc) branched *N*-glycans. This integrin-β1 N-glycosylation pattern was correlated with higher levels of membrane-bound integrin-β1 and also with increased binding to fibronectin. When cultured *in vitro*, tRNAmut cybrids presented lower growth rate than WT cybrids, however, when injected in nude mice, tRNAmut cybrids produced larger tumours and showed higher metastatic potential than WT cybrids. We conclude that mtDNA-driven OXPHOS dysfunction correlates with increased motility and migration capacities, through a mechanism that may involve the cross talk between cancer cell mitochondria and the extracellular matrix.

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Introduction

Metabolic re-wiring, or the ability to adapt cellular metabolism to growth, migration and invasion, has gained considerable attention in the cancer research field over the past decade. A cancer, by definition a proliferating and ultimately invading and metastasizing cell mass, has different nutritional demands than those of most adult tissues, which are mainly in a quiescent state (1). A key feature of the metabolic re-wiring of cancer cells is the high rate of glucose uptake to meet their increased energetic and biosynthetic needs and produce a full-blown tumour (2). Cancer cells often display the so-called 'Warburg effect', i.e. they convert most of the glucose to lactate, rather than metabolizing it through mitochondrial oxidative phosphorylation (OXPHOS), even in the presence of oxygen (3,4). This phenomenon sets mitochondrial activity, in particular OXPHOS, as a central metabolic function for tumour cells. In fact, OXPHOS activity in tumour cells is still controversial, as there are arguments supporting a deficient OXPHOS in tumourigenesis, and others agreeing with an effective utilization of OXPHOS by tumour cells (5). An increasingly accepted notion is that multiple types of tumour bioenergetic signatures occur, ranging from mainly oxidative to exclusively glycolytic, in which OXPHOS activity is significantly reduced (6).

Several mechanisms are able to induce OXPHOS downregulation in cancer, including those associated with the microenvironment (low oxygen tension) and oncogenic pathway activation [where hypoxia inducible factor-1alpha (HIF-1α) appears as a key molecule], as well as those related to genetic alterations in OXPHOS-related genes encoded by the nuclear DNA or mitochondrial DNA (mtDNA). mtDNA mutations have been detected in a wide variety of human tumours and, in some instances, associated with poor prognosis (7–9). Such mutations are often deleterious and result in OXPHOS impairment, thereby altering mitochondrial bioenergetics and biosynthesis (10). There is compelling evidence that mtDNA mutations not only lead to OXPHOS deficiency, but also empower cancer cells with enhanced tumourigenic properties, namely increased capacity to migrate, invade and metastasize (11,12). Nonetheless, there is scant information about the molecular mechanisms and signalling pathways that connect mtDNA mutations/OXPHOS deficiency with migration, invasion and metastization. In the process of migration and invasion, cancer cells bind to molecules of the extracellular matrix (ECM) through the integrin family of transmembrane glycoprotein receptors (13). Integrins are heterodimers composed of α and β subunits and mediate anchorage and migration of cancer cells over a variety of ECM molecules, such as collagen, fibronectin, laminin and vitronectin (13).

In this study, we have generated cybrid cancer cell lines that recapitulate the setting of OXPHOS deficiency (caused by a mtDNA tRNAleu mutation) and normal OXPHOS [with wild-type (WT) mtDNA]. In comparison with control cells, OXPHOS-deficient cells displayed increased motility and migration properties, which were associated with differential glycosylation and membrane localization of integrin-β1, together with increased expression of fibronectin.

Results

Generation of cybrid cell lines

Cybrid (cytoplasmic hybrid) cell lines are hybrid cells where the nuclear and mitochondrial genomes are from different sources (14). Cybrids are a valuable *in vitro* cellular system for the study of mitochondrial gene function, because they allow the comparison of different mtDNA genomes (either WT or mutant) against the same nuclear background.

To create cybrids with OXPHOS dysfunction (hereafter named tRNAmut cybrids), we fused mtDNA-depleted cells (143Bρ0 cells), which were derived from the osteosarcoma cell line 143B, with platelets harbouring the mtDNA A3243T mutation in the tRNAleu (UUR) gene, previously described as pathogenic (15). The platelets were obtained from a patient who was diagnosed with encephalomyopathy and harboured the aforementioned mutation. As controls, we used cybrids that were generated from the fusion of 143Bρ0 cells with WT mtDNA platelets, obtained from a healthy blood donor (hereafter named WT cybrids). The restoration of the expression of the mtDNA-encoded protein cytochrome c oxidase II (COXII) confirms that both WT and tRNAmut cybrids were successfully generated (Fig. 1A). On the other hand, 143Bρo cells, which lack mtDNA, do not express COXII (Fig. 1A), but do show expression of the nuclear-encoded mitochondrial protein succinate dehydrogenase subunit A (SDHA) (Fig. 1B).

As each cell has multiple mtDNA copies, we sequenced the mtDNA to analyse the proportion of mtDNA-mutant molecules, also designated as the degree of heteroplasmy. We determined that tRNAmut cybrids display the A3243T mutation in ~50% of mtDNA molecules (Fig. 1C).

Complete mtDNA sequencing of the 143B cell line and the two cybrids confirmed the absence of mtDNA mutations in 143B and WT cybrids and that the tRNAmut cybrids only harbour the A3243T mutation in the tRNAleu (UUR) (data not shown).

tRNAmut cybrids display decreased oxygen consumption and increased glycolytic rate than WT cybrids

After generating the WT and tRNAmut cybrids, we evaluated the metabolic changes induced by the mtDNA mutation, namely at the level of oxygen consumption (as a measure of OXPHOS function), as well as glucose consumption and lactate production (as a measure of glycolysis). As expected, 143Bρo cells showed almost no oxygen consumption, while tRNAmut cybrids presented a significant decrease in basal oxygen consumption when compared with WT cybrids (Fig. 2A), confirming that the tRNA mutation leads to decreased OXPHOS function. Treatment with the complex V inhibitor oligomycin significantly decreased oxygen consumption in 143B and WT cybrids, but had no effect in 143Bρ0 (Fig. 2B). tRNAmut cybrids were less sensitive to oligomycin, indicating that these cells might have complex V impairment (Fig. 2B). The treatment with carbonyl cyanide 3 chlorophenylhydrazone (CCCP), which forces the mitochondria to work at its maximum respiratory capacity, increased oxygen consumption in 143B, WT cybrids and tRNAmut cybrids but not in 143Bρ0 (Fig. 2B).

The aforementioned differences in oxygen consumption were mirrored by the glycolytic rate of the cell lines. Glucose and lactate levels were quantified in the medium at 0 h and after 96 h of cell culture. Glucose consumption was significantly increased in 143Bρ0 and tRNAmut cybrids when compared with 143B and WT cybrids, respectively (Fig. 3A). Additionally, we observed that 143Bρ0 and tRNAmut cybrids produced more lactate when compared with 143B and WT cybrids, respectively (Fig. 3B).

The combined results of oxygen consumption, glucose consumption and lactate production suggest that the mtDNA tRNA mutation leads to OXPHOS dysfunction and a concomitant increase in glycolytic rate, a phenotype that recapitulates the Warburg effect.

It has been shown that there is a threshold for the positive tumourigenic effects of mtDNA mutations; above this threshold, there is energetic impairment that prevents tumour growth

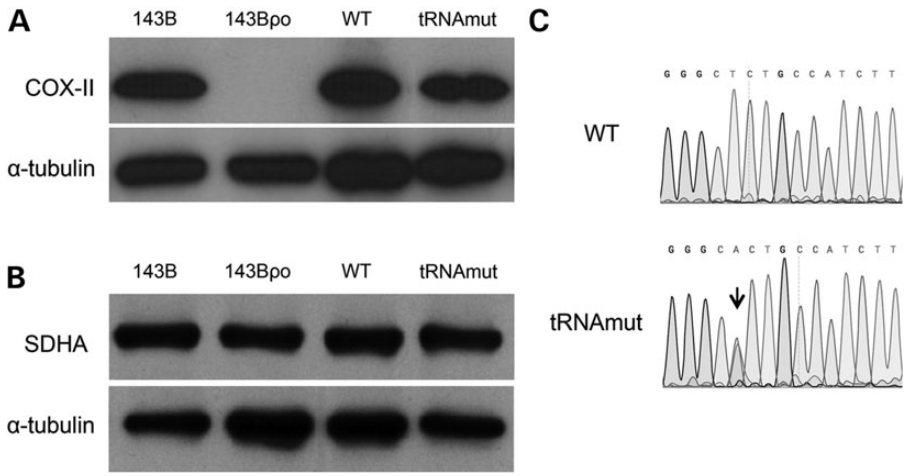

Figure 1. (**A**) Western blot showing that the mtDNA-encoded protein COXII is present in 143B, WT cybrids and tRNAmut cybrids, but absent in 143Bρ0; (**B**) the nuclear-encoded mitochondrial protein SDHA is present in all cell lines. (**C**) Electropherogram showing a stretch of the leucine tRNA gene in WT (top panel) and tRNAmut cybrids (lower panel) and highlighting the A3243T point mutation in the tRNAmut cybrids (arrow; electropherogram shows the reverse sequence of leucine transfer RNA gene).

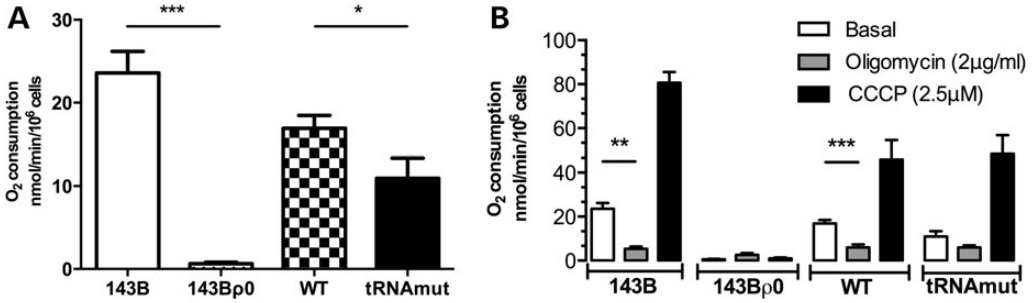

Figure 2. (**A**) Basal oxygen consumption in the four cell lines showing that 143Bρ0 has virtually no mitochondrial respiration and that tRNAmut cybrids have significantly less mitochondrial respiration than WT cybrids. (**B**) Sensitivity to respiration modulators: oligomycin significantly decreased oxygen consumption in 143B and WT cybrids but not in 143Bρ0 or tRNAmut cybrids. Results are representative of at least three independent experiments; error bars are SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(16). We thus decided to focus our analysis in the tumourigenic effects of OXPHOS dysfunction using the tRNAmut cybrids that present 50% of mutation load and compare them with 143B and WT cybrids that have normal mtDNA. Considering that complete lack of mtDNA does not occur in human tumours, 143Bρ0 cells were not further analysed.

Cellular growth and apoptosis are decreased in tRNAmut cybrids

To address the consequences of OXPHOS dysfunction at the level of cellular growth, we counted cells over a period of 5 days and observed that tRNAmut cybrids had significantly lower growth rates than WT cybrids and 143B cells (Fig. 4A). This result indicates that, under optimal growth conditions, cells with OXPHOS deficiency have a decreased growth capacity in comparison with cells with normal OXPHOS.

Apoptosis was evaluated in the cybrid cell lines after treatment with 50 nM staurosporine (STS) [the concentration that most reflected the differences between cell lines (data not shown)]. By determining the geometric mean of the histogram, we confirmed that STS induced apoptosis in all cell lines, although this effect was only statistically significant in 143B (2-fold increase compared with untreated cells). WT cybrids showed a 1.7-fold increase compared with untreated cells, while tRNAmut cybrids were the least sensitive to STS (1.15-fold increase compared with untreated cells) (Fig. 4B and C).

Motility and migration are increased in tRNAmut cybrids

Upon observation of the cell lines under bright field microscopy, there were evident morphological differences between the cell lines: tRNAmut cybrids presented a more spindle-like phenotype and reduced cell–cell contacts, when compared with 143B and WT cybrids (Fig. 5).

Because such morphological differences can denote different cellular migration and motility capabilities, we assessed the distance covered by single cells in the plate (motility) and the rate of closure over time in the wound-healing assay (migration) of the three cell lines, using time-lapse microscopy. In a 14 h period, tRNAmut cybrids showed significantly more individual cell motility than 143B and WT cybrids: tRNAmut cells, on average, covered ~3.3 times the distance of 143B and WT cybrids (Fig. 6A and B). In addition, the wound-healing assay showed that tRNAmut cells had the highest migratory capacity, covering 87% of the wound in 8 h, comparing with WT cybrids (43% of wound coverage) and 143B (35% of wound coverage) (Fig. 6C and D).

tRNAmut cybrids display increased expression and binding to fibronectin

A fundamental step in cancer cell motility and migration is the interaction with the surrounding stroma. Cancer cells often secrete ECM proteins and reshape the surface expression of cell-matrix interacting molecules, such as integrins, to promote their capacity to migrate and invade the adjacent tissues (17).

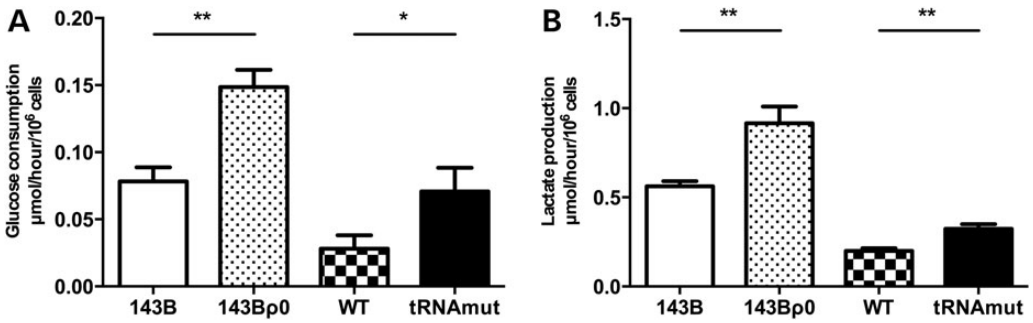

Figure 3. (**A**) Glucose consumption and (**B**) lactate production were assessed by quantifying glucose and lactate, respectively, in the medium of cells cultured for 96 h. Both glucose consumption and lactate production were significantly elevated in 143Bρ0 and tRNAmut cybrids when compared with 143B and WT cybrids, respectively. Results are representative of at least three independent experiments; error bars are SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

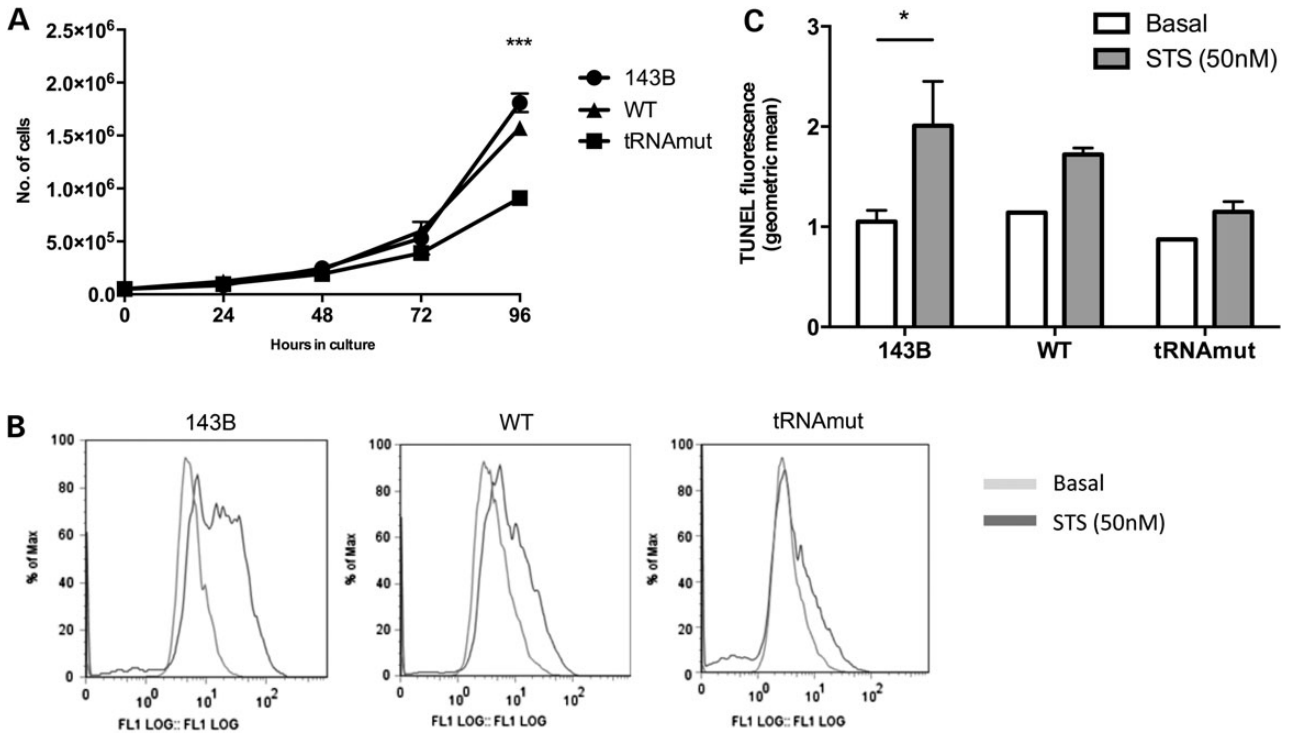

Figure 4. (**A**) Assessment of cell growth and (**B** and **C**) apoptosis *in vitro*. (A) In a period of 96 h, tRNAmut cells showed a slower growth rate than 143B and WT cybrids. (B) Flow cytometry analysis after incubation with 50 nM STS using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Each histogram is representative of three independent experiments. (C) Quantification of TUNEL fluorescence in untreated cells and after STS treatment: tRNAmut cybrids show the lowest response to STS. Results are representative of at least three independent experiments; error bars are SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

To test the binding capacity to different ECM substrates, we performed an adhesion assay by culturing cells in wells coated with collagen type I, collagen type IV, fibronectin or poly-lysine (positive control). We observed that tRNAmut cybrids adhered more efficiently to fibronectin than 143B and WT cybrids (Fig. 7A), while the binding to collagen type I or IV was not significantly altered (Fig. 7B and C, respectively). Furthermore, in western-blot (WB) analysis, tRNAmut cells showed significantly increased levels of fibronectin when compared with WT cybrids and 143B (Fig. 8A and B).

tRNAmut cybrids display increased modification of integrin-β1 with β-1,6 GlcNAc branched *N*-glycans structures

Considering that tRNAmut cybrids show higher motility, migration and binding capacity to fibronectin than 143B and WT cybrids, we addressed the status of integrin-β1 in the three cell lines. Integrins are known to play a crucial role in adhesion to the ECM, thereby contributing to migration, invasion and metastization of tumour cells (18). Integrin-β1 is one of the major players in these processes and is known to preferentially bind to fibronectin after dimerization with integrin-α5 (19).

In the WB analysis, integrin-β1 showed two distinct bands: an upper band with ~130 kDa and a lower band of ~115 kDa (Fig. 8A). Although the overall expression of integrin-β1 did not differ between the three cell lines (Fig. 8C), there was a clear difference in the relative intensity of the upper and lower band: while the parental 143B displayed similar intensity of both bands, the WT cybrids have more expression of 115 kDa integrin-β1 and tRNAmut cybrids were enriched in the 130 kDa band (Fig. 8D). In addition, the integrin-β1 130 kDa band of tRNAmut cybrids also showed less mobility in the gel than the same band of 143B and WT cybrids (Fig. 8A).

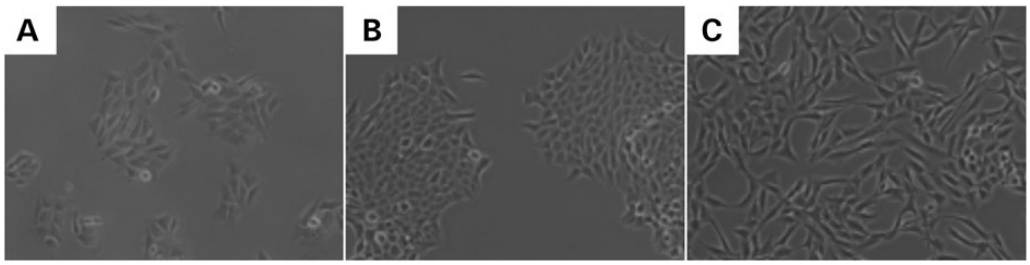

Figure 5. Light microscopy images of 143B (**A**), WT cybrids (**B**) and tRNAmut cybrids (**C**). The tRNAmut cybrids display reduced cell–cell contacts, showing a more spindle-like morphology than 143B and WT cybrids. Pictures were taken using the 20× objective.

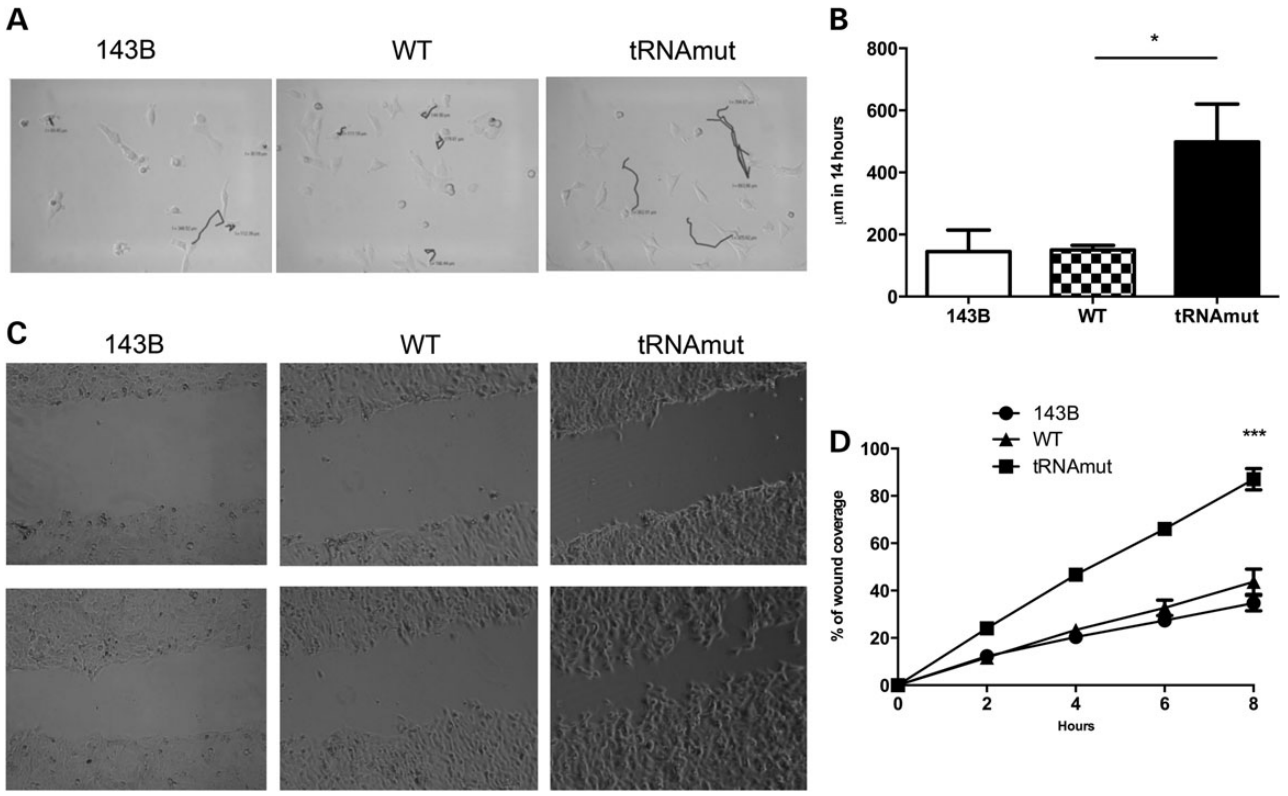

Figure 6. Time-lapse microscopy was used to evaluate motility (distance covered by single cells in a 14 h period; **A** and **B**) and migration (wound-healing assay in a 14 h period; **C** and **D**). In (A), the lines show the distance covered by single cells. In (C) the top panel represents the wound at baseline and the bottom panel represents the wound after 14hrs. tRNAmut cybrids showed significantly increased motility and migration when compared with 143B and WT cybrids. Each image is representative of three independent experiments. Results are representative of at least three independent experiments; error bars are SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Glycosylation modifications have been described to be a key regulatory mechanism of several cell biology processes, including cell adhesion and cell-matrix interaction, particularly affecting the integrin-mediated cellular migration (20,21). Taking into account that integrin-β1 is modified by N-glycosylation and that glycosylation affects the protein mobility in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, we characterized the integrin-β1 N-glycosylation profile in each cybrid cancer cell lines using two different *N*-glycosidases: endoglycosidase H (Endo H), which is an endoglycosidase that cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins, and peptide-*N*-glycosidase F (PNGase F), which is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. In 143B and WT cybrids, Endo H digestion completely converted the 115 kDa band to an 85 kDa band (corresponding to the size of integrin-β1 core protein), while in tRNAmut cybrids, Endo H only yielded a faint digestion product (Fig. 9A). On the other hand, PNGase F digestion converted the 130 and 115 kDa bands to the 85 kDa-sized integrin-β1 in 143B, WT cybrids and tRNAmut cybrids (Fig. 9A). These results indicate that, in 143B and WT cells, integrin-β1 is modified with high mannose, hybrid and complex type *N*-glycans, whereas in tRNAmut cybrids, integrin-β1 is mainly modified with complex type *N*-glycans. To determine whether integrin-β1 from tRNAmut cells are modified with the β-1,6 GlcNAc branched structures catalysed by beta1,6 *N*-acetylglucosaminyltransferase V (GnT-V) enzyme, we performed integrin-β1 immunoprecipitation followed by β-1,6 GlcNAc branched *N*-glycans recognition using the phaseolus vulgaris leucoagglutinin lectin (L-PHA). The increased L-PHA reactivity in tRNAmut cybrids, in comparison with 143B and WT

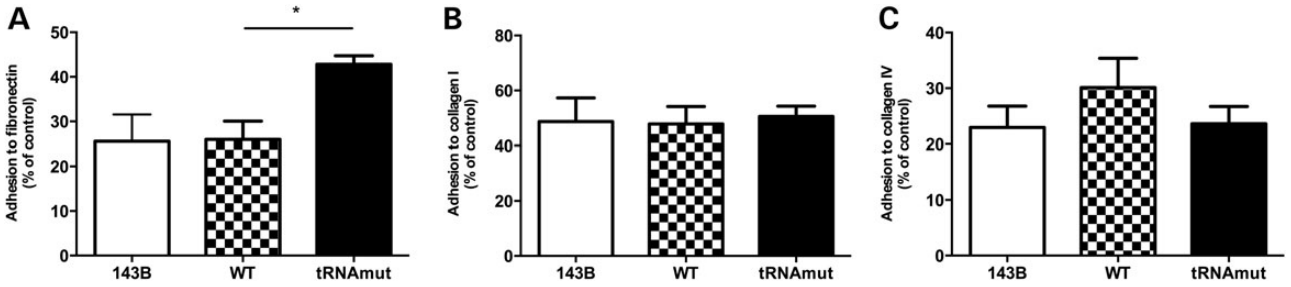

Figure 7. Quantification of cell adhesion to fibronectin (A), collagen type I (B) or collagen type IV (C), shown as percentage of adhesion to poly-lysine (positive control). tRNAmut cybrids adhere more efficiently to fibronectin than 143B or WT cybrids, while no differences were observed concerning collagen type I or type IV. Results are representative of at least three independent experiments; error bars are SEM; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

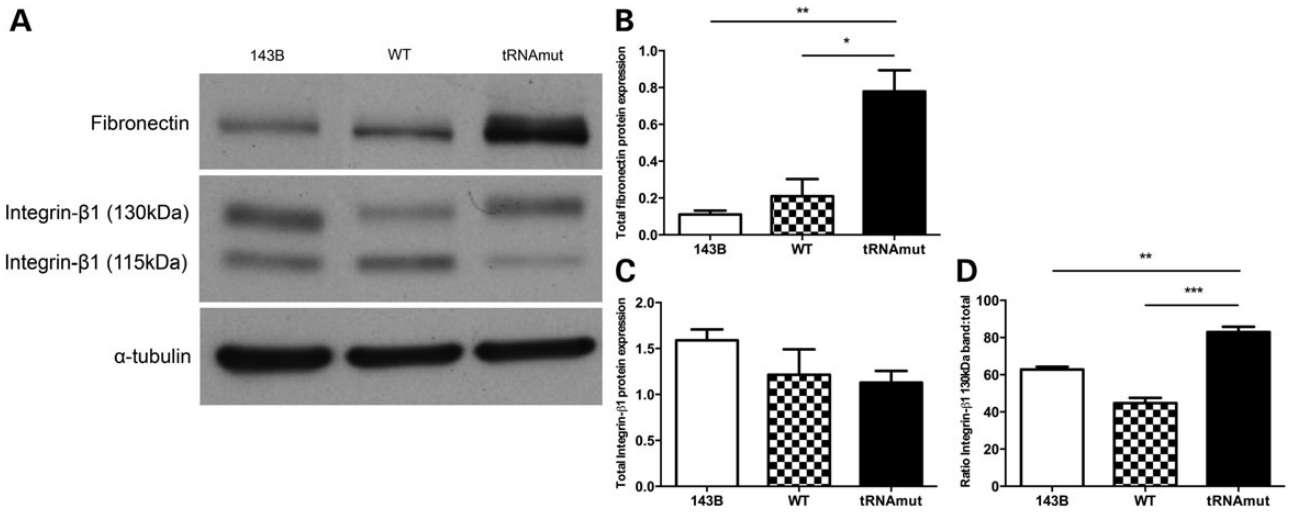

Figure 8. Western blots showing the expression of fibronectin and integrin-β1. (A) Representative WB showing increased fibronectin expression and enrichment of the integrin-β1 130 kDa in tRNAmut cybrids. The quantification of fibronectin (B), total integrin-β1 (C) and the ratio 130 : 115 kDa bands of integrin-β1 (D) is shown. Results are representative of at least three independent experiments; error bars are SEM; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

cells (Fig. 9B and C), demonstrates that tRNAmut cybrids are enriched in GnT-V-mediated β-1,6 GlcNAc branched structures (~3.5-fold higher than WT cybrids).

tRNAmut cybrids show predominantly integrin-β1 and integrin-α5β1 membrane localization

We next assessed whether the differences in integrin-β1 glycosylation had an impact on its sub-cellular localization, as it had been previously shown (22). Using immunofluorescence staining, tRNAmut cybrids showed predominantly membrane-localized integrin-β1, in structures that resembled focal adhesions (Fig. 10A, upper-right picture). On the other hand, 143B and WT cybrids (Fig. 10A, upper-left and upper-middle pictures, respectively) showed mostly cytoplasmic staining. To quantify the levels of membrane-bound integrin-β1, we have used imaging flow cytometry that enables the quantification of proteins in different cellular compartments. Applying a setting that specifically quantifies the levels of membrane-localized integrin-β1, we saw that the tRNAmut cybrids displayed higher levels of membrane-bound integrin-β1 than 143B and WT cybrids (Fig. 11A). In addition, after categorizing the expression levels of membrane-bound integrin-β1 as low or high (Fig. 11E), we observed that, in the tRNAmut cybrids, the percentage of cells displaying strong expression of membrane-bound integrin-β1 was increased in comparison with 143B or WT cybrids (Fig. 11B). Conversely, 143B or WT cybrids had a higher percentage of cells displaying low expression of membrane-bound integrin-β1 than tRNAmut cybrids (Fig. 11B).

Because integrin-β1 requires dimerization with α subunits for proper function, we have used the same imaging flow cytometry analysis to quantify the levels of membrane-bound integrin-α5β1, the dimer that preferentially binds fibronectin. Similarly to what we observed for integrin-β1, the levels of membrane-bound integrin-α5β1 were elevated in tRNAmut cybrids, when compared with 143B and WT cybrids (Fig. 11C). tRNAmut cybrids also showed a higher percentage of cells with a higher expression of membrane-bound integrin-α5β1 and lower percentage of cells with low expression, when compared with 143B and WT cybrids (Fig. 11D).

Phenformin, a complex I inhibitor, mimics the effects of the tRNA mutation

Phenformin is a selective OXPHOS complex I inhibitor (23). We have tested the effects of phenformin in WT cybrids and found that it inhibited O_2 consumption to a degree similar to that of oligomycin (Fig. 10C). To evaluate whether phenformin-induced OXPHOS dysfunction in 143B and WT cybrids had an impact on integrin-β1 localization, we treated cells with 5 mM of phenformin for 1 h and evaluated the effects by confocal microscopy and imaging flow cytometry. Using

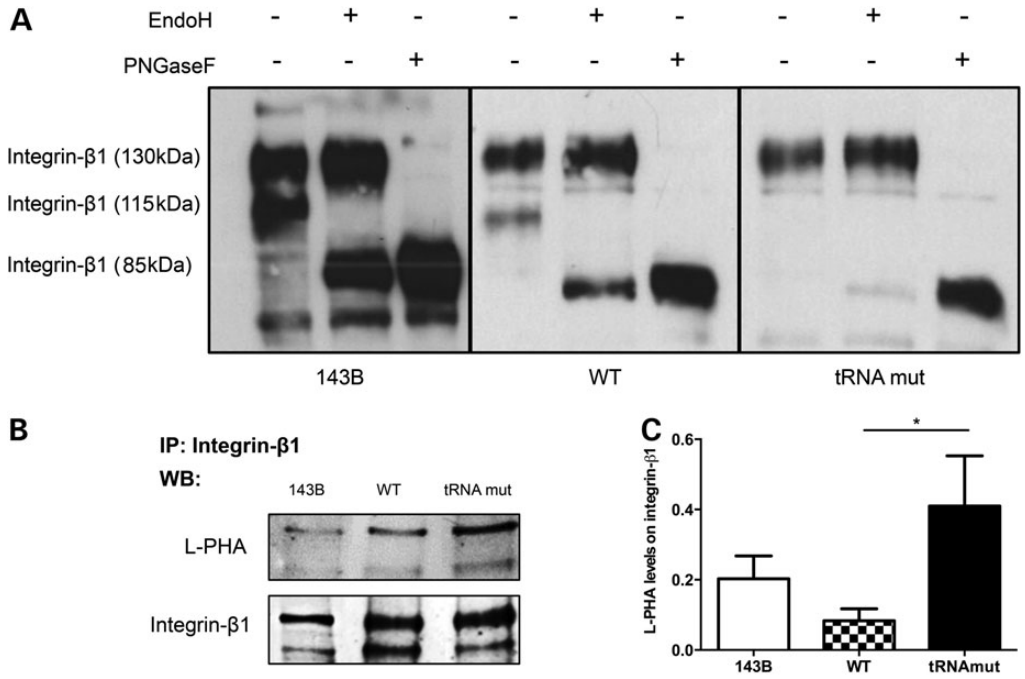

Figure 9. (**A**) Sensitivity to the glycan-cleaving enzymes Endo H (cleaving only high-mannose type glycans) and PNGase F (cleaves all N-linked glycans). 143B and WT, but not tRNAmut cybrids were sensitive to treatment with Endo H, while all cell lines were sensitive to PNGase F. (**B**) Integrin-β1 immunoprecipitation followed by β-1,6 GlcNAc branched *N*-glycans recognition (using L-PHA lectin). tRNAmut cybrids show significantly increased levels of β-1,6 GlcNAc branched *N*-glycan structures in integrin-β1 in comparison to WT cybrids (**C**). Results are representative of at least three independent experiments; error bars are SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

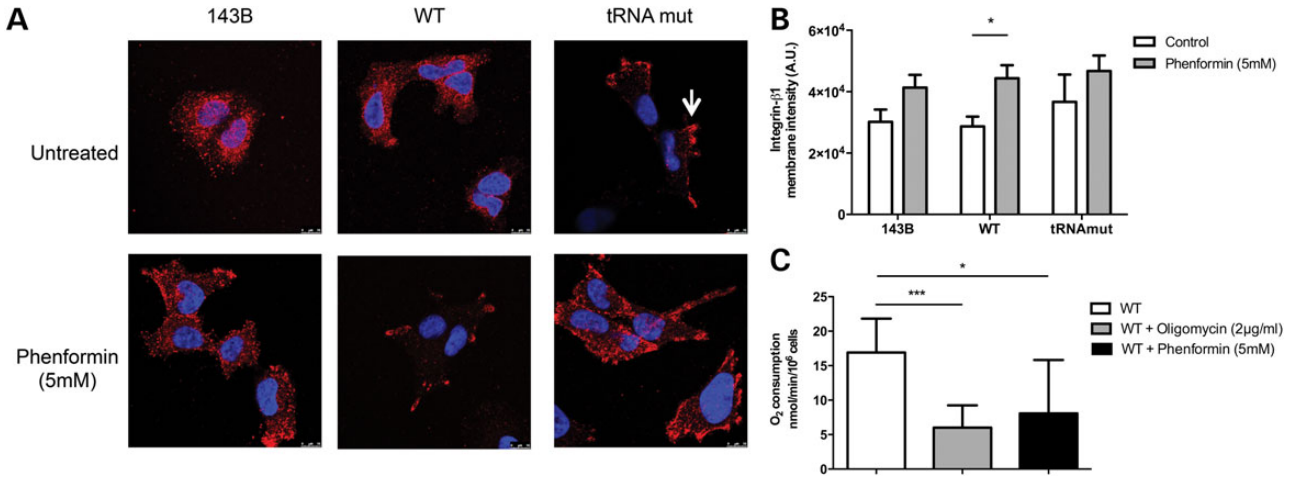

Figure 10. (**A**) Confocal microscopy using anti-integrin-β1 antibody (red). Untreated 143B and WT cells displayed integrin-β1 expression mainly localized in the cytoplasm, while tRNAmut cybrids showed integrin-β1 mainly in membrane protrusions, in structures that resembled focal adhesions (arrow). Further, 5 mM phenformin treatment in 143B and WT cybrids induced an integrin-β1 expression pattern that mimicked that found in untreated tRNAmut cybrids. Nuclei are stained in blue [4',6-diamidino-2-phenylindole]. Pictures were taken using the 63× objective. (**B**) Quantification of membrane-bound integrin-β1 by imaging flow cytometry in cells treated with 5 mM phenformin, showing that the levels of membrane-bound integrin-β1 increase in all cell lines, although only significantly in WT cybrids. (**C**) Phenformin treatment significantly reduces oxygen consumption in WT cybrids. Results are representative of at least three independent experiments; error bars are SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

confocal microscopy, 143B and WT cybrids showed membrane re-localization of integrin-β1 with a pattern that closely resembled that of tRNAmut cybrids, whereas phenformin had little effect on integrin-β1 localization in tRNAmut cybrids (Fig. 10A, bottom panels). After quantification by imaging flow cytometry, we confirmed that phenformin increased the levels of membrane-localized integrin-β1 in all cell lines, although only reaching the level of statistical significance in WT cybrids (Fig. 10B). Such effect was less evident in the tRNAmut cybrids that intrinsically have OXPHOS dysfunction and present higher levels of membrane-bound integrin-β1 (Fig. 10B).

tRNAmut cybrids have higher tumourigenic potential in nude mice than WT cybrids

Taking into consideration that a metabolic defect might have considerably different effects depending on the microenvironment, we assessed the tumour-forming capacity of WT and tRNAmut cybrids *in vivo*. One million cells were injected in the

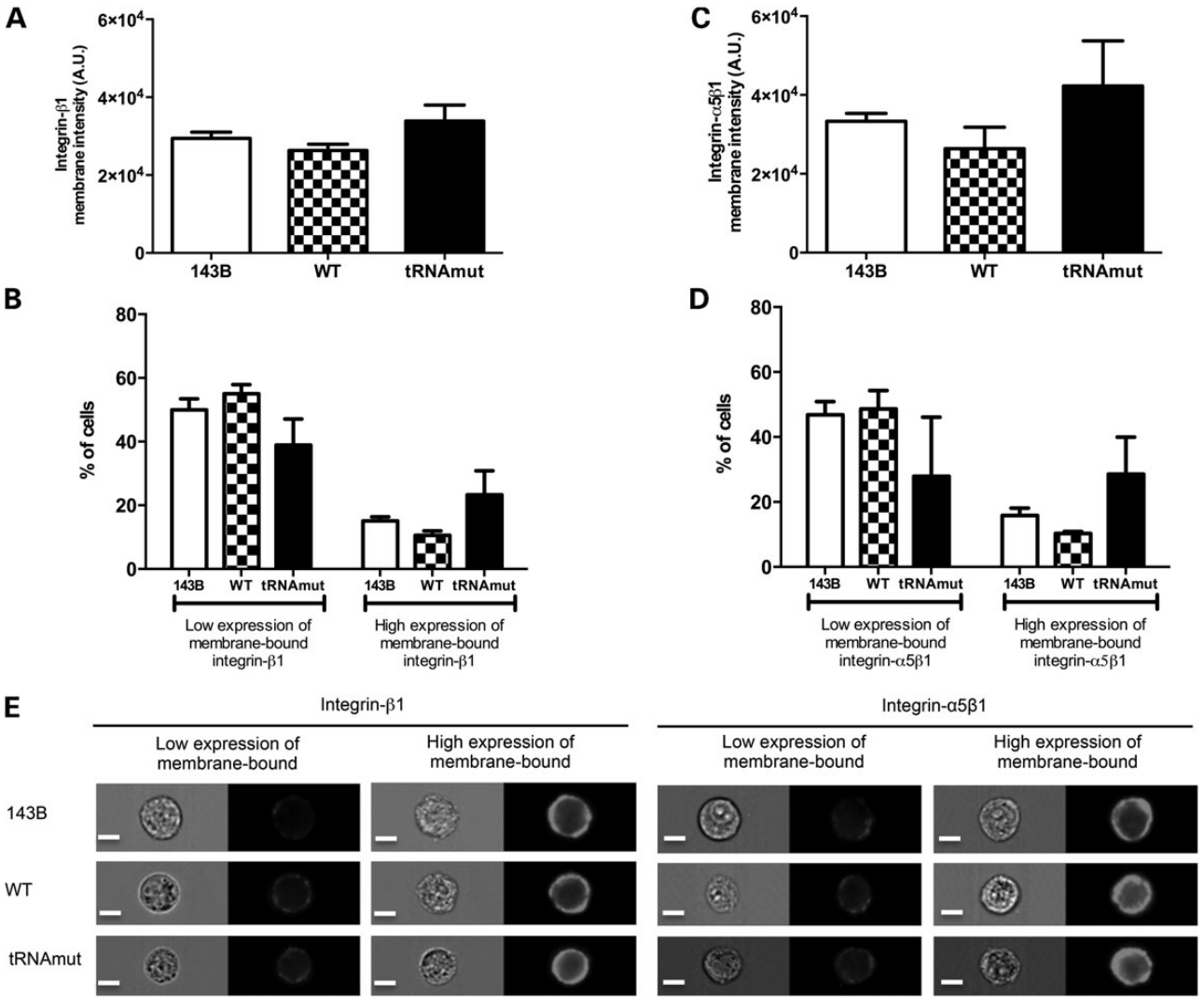

Figure 11. Imaging flow cytometry quantification of the membrane-bound integrin-β1 and membrane-bound integrin-α5β1. Graphics (**A**) and (**C**) illustrate the mean expression level of membrane-bound integrin-β1 and membrane-bound integrin-α5β1, which are increased in tRNAmut cybrids when compared with 143B and WT cybrids. In graphics (**B**) and (**D**), we sub-divided the cell population into those with low or those with high expression of membrane-bound integrin-β1 or integrin-α5β1. tRNAmut cybrids have an increased proportion of cells with high expression levels of membrane-bound integrin-β1 (B) and integrin-α5β1 (D); conversely, 143B and WT cybrids have increased percentage of cells with low levels of membrane-bound integrin-β1 (B) and integrin-α5β1 (D). Results are representative of at least three independent experiments; error bars are SEM. For the three cell lines, representative bright-field images and the corresponding fluorescent image from the low or high expression membrane-bound integrin-β1 or integrin-α5β1 cell populations acquired with the imaging flow cytometer are shown in (**E**). Scale bar 10 μm.

dorsal flank of nude mice and tumour growth was monitored for ~36 days. While tumour formation was observed in all mice injected either with WT or with tRNAmut cybrid cells (except one mouse injected with the WT cybrid), the tumours derived from tRNAmut cybrids were significantly larger in volume than tumours derived from WT cybrids (Fig. 12A). In addition, we observed metastatic foci in the lungs of 3 of the 14 mice injected with tRNAmut cybrids, while no animal injected with WT cybrids had lung metastases (data not shown). To further assess the metastatic potential of WT and tRNAmut cybrids, we repeated the *in vivo* tumour-forming capacity experiment, but instead of sacrificing the animals by Day 36, the tumours were removed and the mice were kept alive until Day 49 (13 days after tumour removal) to allow the formation of metastases. After collecting the lungs of the animals, we performed immunohistochemistry against vimentin (a marker expressed by WT and tRNAmut cybrids) and found that 67% of mice injected with tRNAmut cybrids displayed lung metastases, whereas only 33% of mice injected with WT cybrids had lung metastases (Fig. 12B and C).

Discussion

In his seminal works, Warburg postulated that deficient OXPHOS was on the origin of tumourigenesis, owing to his consistent observations of lack of respiration and increased lactate production in human tumours (3). Because this phenotype persisted even in the presence of oxygen, Warburg designated it as aerobic glycolysis. Several lines of evidence have confirmed that OXPHOS deficiency is common to many human tumours, although its functional role in tumour development remains under study.

The consequences of mitochondrial OXPHOS dysfunction in cancer cells have been addressed by various approaches, namely chemical inhibition (using OXPHOS-targeted drugs, such as oligomycin, rotenone or phenformin) or genetic manipulation, via

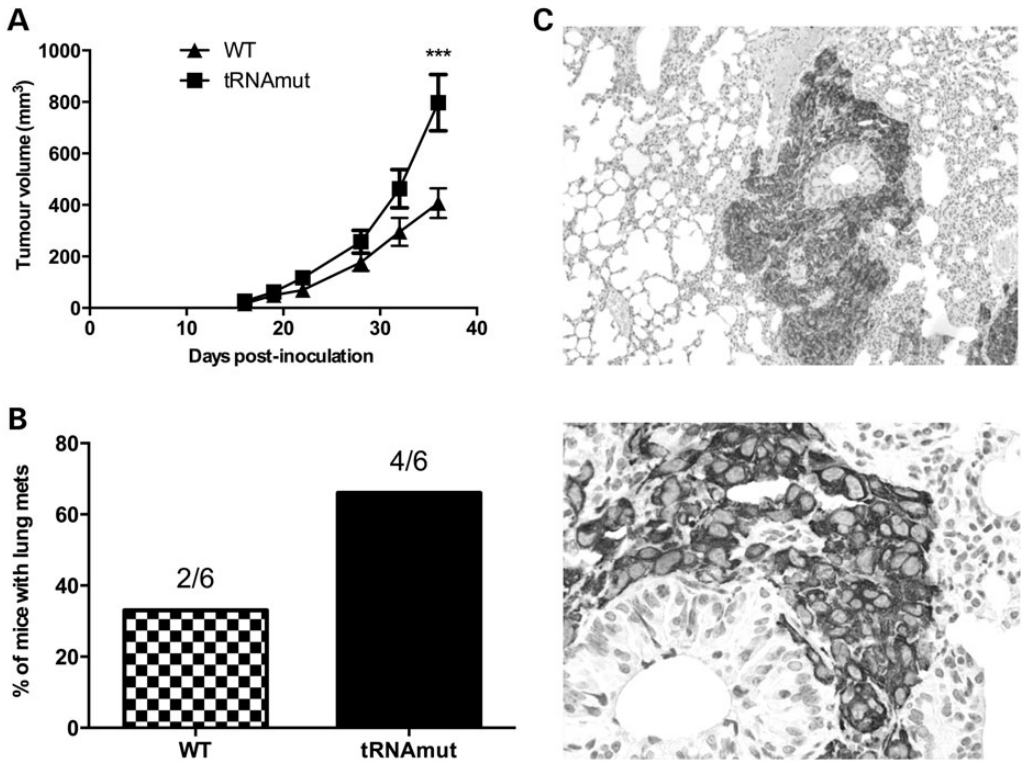

Figure 12. *In vivo* tumour growth and metastatic capacity of WT and tRNAmut cybrids. After injecting in the dorsal flank of nude mice, tRNAmut cybrids gave rise to larger tumours than WT cybrids (**A**). Metastatic potential (**B**) was assessed by injecting cells in the dorsal flank of nude mice, removing the tumours (~Day 36) and euthanizing mice by Day 49. The analysis of the whole lungs revealed that 67% of the animals injected with tRNAmut cells presented lung metastases, while only 33% of those injected with WT cybrids showed lung metastases. (**C**) Illustration of a lung metastasis in an animal injected with tRNAmut cybrids. Pictures were taken using the 10× objective (top image) and the 40× objective (bottom image).

the introduction of mtDNA mutations (cybrid cell lines). mtDNA mutations are found in a wide variety of cancers, such as colorectal, thyroid, gastric, breast, leukaemia, glioblastoma and prostate (10,24–28). Many of such mutations are deleterious and able to inhibit OXPHOS, implying that alterations in mitochondrial bioenergetics and metabolism have a role in supporting neoplastic transformation. An interesting finding is that there is no mutational hotspot within mtDNA; instead, mtDNA mutations are scattered across the mitochondrial genome, spanning all the OXPHOS-encoding genes, suggesting that the key target is OXPHOS function, rather than a specific gene.

Following the genetic-based strategy, we created a cybrid cell model, where we engineered cells to have WT mtDNA (WT cybrids) or a pathogenic mtDNA mutation (tRNAmut cybrids). Given the diversity of mtDNA mutations found in human cancers, we chose to use a deleterious mutation (A3243T in the leucine tRNA) that clearly induces OXPHOS dysfunction (15). In this way, our model is well fitted to address the converging outcome of mtDNA mutations, i.e. OXPHOS dysfunction. In addition, this mutation occurs in the same nucleotide as the A3243G, which has already been detected in human cancers (29,30). Our results showed that tRNAmut cybrids exhibited OXPHOS dysfunction, because they display lower oxygen consumption when compared with WT cybrids. This is in line with a previous report showing that the same germline mutation caused decreased activity of complexes I, III and IV in the muscle fibres of an encephalomyopathy patient (15). Furthermore, tRNAmut cybrids showed a metabolic shift towards aerobic glycolysis, as evidenced by the increased glucose consumption and lactate production. This cybrid model thus constitutes a valuable tool to study the phenotypic effects of OXPHOS dysfunction.

The influence of the microenvironment over tumour cell behaviour is an important aspect for studying the relevance of altered metabolism in tumourigenesis. When we compared growth capacity *in vitro*, tRNAmut cybrids showed slower growth rate than WT cybrids. However, when injected in the dorsal flank of nude mice, tRNAmut cybrids produced larger and faster-growing tumours. These results are in accordance with previous studies using cybrid cells (31–33) and fit with numerous examples where the *in vivo* results diverge from the results obtained *in vitro*. It is possible that, within the microenvironment where the cells are injected, the nutrient availability, presence of fibroblasts and immune cells may provide growth advantage to OXPHOS-deficient cells, while optimal *in vitro* growth conditions benefit OXPHOS-proficient cells. Moreover, tRNAmut cybrids seem to be more resistant to the apoptosis inducer STS, a finding that could also explain the increased size of the tumours produced by tRNAmut cybrids in nude mice. Several studies showed that mtDNA mutations suppress apoptosis in cultured cybrids (32,33), which is in accordance to the crucial role played by mitochondria and the respiratory chain in apoptosis (34).

WT and tRNAmut cybrids showed marked differences concerning motility and migration: tRNAmut cybrids displayed higher individual cell motility (when plated in low density) and migration (wound healing) than WT cybrids. These differences were associated with distinct cell morphology: WT cybrids formed colonies with clear cell–cell interactions, whereas tRNAmut cybrids appeared as individualized (dispersed) cells with few cell–cell

contacts. Noteworthily, when injected in nude mice, tRNAmut cybrids produced more lung metastases than WT cybrids, suggesting that the *in vitro* migratory capacities of tRNAmut cybrids were associated with an increased metastatic ability *in vivo*.

Our results fit with previous findings suggesting that OXPHOS dysfunction increases the migratory and metastatic potential of cancer cells (11,12,33). In some of these studies, reactive oxygen species (ROS) were pointed out as important mediators of the enhanced migration and metastization of OXPHOS-deficient tumour cells (12), although the underlying effector molecules remain to be clarified.

OXPHOS dysfunction has been advanced to modulate the ECM in a way that promotes cell invasion and metastatic properties (35,36). Crucial for migration and invasion, the crosstalk cancer cell-stroma is mediated by adhesion proteins, where integrins play a central role by regulating a diverse array of cellular functions necessary to the initiation, progression and metastization of solid tumours (18). Surface integrin-β1 promotes cell migration and invasion by binding to fibronectin in the ECM. In our experiments, tRNAmut cybrids showed both an increased expression and binding to fibronectin compared with 143B and WT cybrids. Interestingly, while no significant changes were observed in the total levels of integrin-β1, tRNAmut cybrids consistently exhibited higher levels of the 130 kDa integrin-β1 isoform than 143B and WT cells.

Integrins are glycoproteins and therefore major carriers of *N*-glycans (sequences of carbohydrates attached to asparagine residues of the protein). Glycosylation has been described as a fundamental molecular mechanism affecting the folding, expression, intracellular trafficking, localization, activity and half-life of several proteins, both in normal and pathological conditions (37,38). Integrin-β1 is tightly regulated by N-glycosylation. Indeed, deglycosylation mutants of integrin-β1, or treatment with the N-glycosylation inhibitor tunicamycin, abolish integrin-β1 transport to the cell surface and its binding to ECM substrates (21). Moreover, aberrant N-glycosylation with increased expression of β-1,6 GlcNAc branched *N*-glycans (catalysed by GnTV enzyme) directs more integrin-β1 delivery to plasma membrane and promotes fibronectin-based cell migration and invasion (39). Such β-1,6 GlcNAc branched glycans in integrin-α5β1 have been shown to be associated with increased cell spreading on fibronectin in a context of oncogenic signalling driven by mutant Ras (40).

Our results show that integrin-β1 N-glycosylation pattern differs between WT and tRNAmut cybrids, specifically in the levels of integrin-β1 modified with the β-1,6 GlcNAc branched *N*-glycans that were significantly elevated in tRNAmut cybrids. In line with previous reports (39,40), such integrin-β1 N-glycan modification in tRNAmut cybrids may account for their increased binding to fibronectin, as well as for enhanced motility and migration capabilities.

Our observation of differential integrin-β1 N-glycosylation levels between tRNAmut and WT cybrids may be associated with (i) increased expression or enzymatic activity of GnT-V or (ii) increased availability of the substrates for N-glycosylation such as UDP-N-acetylglucosamine (UDP-GlcNAc), promoting an increased N-glycosylation biosynthetic pathway. The production of UDP-GlcNAc for N-glycosylation is achieved by the hexosamine biosynthetic pathway, which requires the coordination of both glucose and glutamine metabolism. Indeed, UDP-GlcNAc levels have been shown to be critical factors in the production of β-1,6-branched *N*-glycans, as they increase the production of these sugar chains without an increase in GnT-V activity (41). While our data associate OXPHOS deficiency to increased glucose uptake, it has also been observed that glutamine metabolism is essential for cancer cells harbouring mtDNA mutations (42). Taking all this together, we speculate that OXPHOS deficiency may remodel glucose and glutamine metabolism, thereby affecting the intracellular pool of N-glycosylation substrates and leading to the modification of the N-linked glycosylation of membrane proteins like integrin-β1. Interestingly, Wellen *et al.* (43) demonstrated that glucose availability to the hexosamine pathway regulates interleukin-3 receptor alpha (IL-3Rα) surface expression in a N-linked glycosylation-dependent manner. Our finding on the association between carbohydrate maturation on integrin-β1 and the glycolytic shift caused by OXPHOS deficiency thus support and extend the results of Wellen *et al.* (43), strengthening the notion that cellular metabolism can affect signal transduction through regulation of glycosylation.

Hung *et al.* (44) had advanced a connection between OXPHOS deficiency and integrin signalling in a model of gastric cancer cells. Hung *et al.* (44) used a model of chemically induced OXPHOS dysfunction (using the mitochondrial inhibitors oligomycin and antimycin A) and proposed that mitochondrial dysfunction enhances migration through mitochondria-generated ROS-mediated integrin-β5 expression. We focussed mainly on the effects elicited by genetically induced OXPHOS dysfunction, but we also observed that treatment with the mitochondrial complex I inhibitor phenformin resulted in increased surface expression of integrin-β1. Our results, combined with those of Hung *et al.* (44), suggest that mitochondrial dysfunction is associated with integrin-mediated cell motility, migration and invasion.

In conclusion, we have built an *in vitro* cybrid cell model that recapitulates OXPHOS dysfunction of tumour cells induced by mtDNA mutations. In this study, we show that OXPHOS dysfunction has a major impact in the migratory phenotype of cancer cells *in vitro*, and that the adhesion glycoprotein integrin-β1 may be behind the mechanism by which the defect in OXPHOS leads to an increased motility and migration capacity. In addition, when transplanted in nude mice, tRNAmut cybrids display increased tumourigenic/metastatic potential than WT cybrids.

Materials and Methods

Cell lines

Cell lines were cultured in Dulbecco's modified Eagle's medium high-glucose supplemented with 10% (v/v) inactivated foetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin (all from GIBCO, Life Technologies, NY, USA) and 50 μg/ml uridine (Sigma-Aldrich, St. Louis, USA). Cells were maintained at 37°C, 5% CO_2 in a humidified incubator and cultured as a monolayer.

The 143Bρ0 is an mtDNA-depleted cell line, derived from 143B osteosarcoma cells after transient expression of UL12.5 *Herpes simplex* protein (45). Compared with the alternative method of generating ρ0 cells—long-term exposure to ethidium bromide—this method of mtDNA depletion has the advantage of selective degradation, assuring that no alterations are induced in the nuclear DNA.

Cybrid cell lines were obtained after fusion of 143Bρ0 cells with human platelets harbouring either WT or mutant mtDNA. Platelets were isolated from peripheral blood collected in ethylenediaminetetraacetic acid (EDTA) tubes. Whole blood was mixed with one-tenth (v/v) of a 10× warm salt solution of citrate (0.15 M NaCl and 0.1 M trisodium citrate dehydrated, pH 7.0), and centrifuged for 20 min at 200*g*. To pellet the platelets, the top three-fourths of the platelet-rich plasma (supernatant) were centrifuged for 20 min at 1500*g*. Platelets were resuspended in

physiological saline (0.15 M NaCl, 15 mM Tris–HCl, pH 7.4) as previously described (46).

After isolation, the platelet suspension was centrifuged for 15 min at 1500*g* and the supernatant discharged. One million 143Bρ0 cells were carefully added to the platelets' pellet, which was then centrifuged for 10 min at 180*g*. The cellular fusion was achieved when the pellet was resuspended in 0.1 ml of PEG 45% (v/v) and incubated for 1 min. The fusion mixture was then plated in Petri dishes in different dilutions (1 : 1, 1 : 10 and 1 : 100) and selection began 48 h later, by removing uridine from the culture medium. Only the clones that survive and proliferate after uridine removal were selected.

Western blot analysis

Cells were detached with Versene dissociation solution (GIBCO) and lysed in RIPA buffer (50 mM Tris–HCl, 1% NP-40, 15 mM NaCl and 2 mM EDTA, pH 7.5) supplemented with protease (Roche Applied Science, Penzberg, Germany) and phosphatase inhibitors (Sigma-Aldrich). Protein extracts (25–100 μg) were denatured, resolved in SDS-PAGE gels and then electrotransferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, USA) for 2 h at 100 V and 4°C, or alternatively overnight at 30 V and 4°C.

Membranes were blocked for 1 h at room temperature [in phosphate buffered saline (PBS) containing 0.5% Tween-20 and 5% low-fat dry milk] and incubated with primary antibodies diluted in blocking solution, according to the manufacturer's instructions. The primary antibodies used were the following: α-tubulin (Sigma-Aldrich); COXII (Mitosciences, Abcam, Cambridge, UK); SDHA (Mitosciences); fibronectin (Santa Cruz, Dallas, USA); integrin-β1 (BD Transduction Laboratories, San Jose, USA). Membranes were then washed with PBS-T and incubated with the suitable horseradish peroxidase conjugated secondary antibody. Protein bands were detected by chemiluminescense and X-ray film exposure (GE Healthcare).

Measurement of oxygen consumption

Cells were trypsinized and resuspended in FBS-containing culture medium. Cells were counted and aliquots containing 5×10^6 cells were further pelleted, resuspended in Krebs buffer containing 132 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 6 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and allowed to equilibrate for 5 min in an oxygen electrode chamber (DW1, Clark electrode, Hansatech, Norfolk, UK), after calibration for dissolved oxygen. After recording the basal rate of oxygen consumption (in nmol/ml/min), maximum respiration was assessed by adding 2.5 μM CCCP plus 2 μg/ml oligomycin to the reaction medium. Potassium cyanide (700 μM) was added at the end of the experiment to confirm O_2 consumption by mitochondria.

Glucose and lactate quantification

Identical cell numbers (10^4) were plated in 6-well plates and the medium was collected at 0 h and after 96 h in culture. Glucose levels present in the conditioned medium after 96 h in culture were quantified using the Glucose GOD/PAP Kit (Roche Applied Science) and subtracted to the initial levels (0 h). Lactate was quantified in a similar manner, using the lactic oxidase-peroxidase (LO-POD) enzymatic colorimetric assay (Spinreact, Sant Esteve de Bas, Spain).

Cell population growth and apoptosis

For cell population growth studies, identical cell numbers (10^4) were plated in 6-well plates and cells were counted in 24 h intervals, for 96 h, using a Z Series Particle Count and Size Analyser (Beckman Coulter, Brea, USA).

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay ['*In situ* cell death detection kit, fluorescein' (Roche Applied Science)] was used to evaluate and quantify apoptosis induced by STS. Cells were treated for 4 h with three concentrations—25, 50 and 100 nM—of STS in dimethyl sulfoxide and the assay was performed according to the manufacturer's protocol. The fluorescence was detected in the range of 515–565 nm by flow cytometry (Beckman Coulter). Analysis of the results was performed in FlowJo software (Tree Star) and the geometric mean of the curves was calculated. Whenever necessary, a gate was drawn to exclude unspecific fluorescence.

Motility and migration

For individual motility assays, cells were plated in low density (10^4 cells) in a 12-well plate. After seeding for 24 h, cells were monitored for 14 h using a Leica DMI 6000B time-lapse microscope (Leica Microsystems, Wetzlar, Germany). The movement covered by a single cell was quantified only if the cell was clearly individualized, remained in the field and did not enter division or apoptosis. For migration (wound healing) assays, cells were plated in high density (5×10^5 cells), to reach confluence. After seeding for 8 h, the cell monolayer was scratched with a pipette tip, cell debris was removed by replacing the culture medium and cells were monitored in a Leica DMI 6000B time-lapse microscope (Leica Microsystems) using the 10× objective. Migration was defined as the capacity to migrate into the wound and measured as the percentage of wound coverage through time. For both assays, images were automatically collected in each field every 5 min using LAS AF software (Leica Microsystems) and further processed using the same software. Appropriate groups of images corresponding to the same field were joined to make the film.

PNGase F and Endo H digestion

Total cell lysates (30 μg) were combined with denaturing buffer and incubated at 100°C for 10 min. Samples were then digested with 1 unit of PNGase F or Endo H (New England Biolabs, Ipswich, USA) overnight at 37°C. The deglycosylated proteins were loaded in SDS-PAGE gels and immunoblotted with anti-integrin-β1 antibody (BD Transduction Laboratories). For controls, samples were incubated without the enzymes.

Integrin-β1 immunoprecipitation and lectin blot analysis

Equal amounts of total protein (1500 μg) from each cell lysate were pre-cleared with protein G-sepharose beads (GE Healthcare) for 1 h and the supernatant was incubated overnight with 5 μg of monoclonal antibody against integrin-β1 (BD Transduction Laboratories). After that, incubation with protein G-sepharose for 2 h was performed and the immune complexes were released by boiling for 5 min at 95°C in Laemmli buffer. Protein extracts were resolved in SDS-PAGE gels, electrotransferred onto a nitrocellulose membrane (GE Healthcare) for 2 h at 100 V and then probed with the primary antibody against integrin-β1 (BD Transduction Laboratories). For β-1,6 GlcNAc branched structure analysis on integrin-β1 immuno-precipitated, membranes were probed with biotinylated *Phaseolus vulgaris* leucoagglutinin lectin

(L-PHA), a lectin that specifically recognizes the β-1,6 GlcNAc branched N-glycan structure catalysed by *N*-acetylglucosaminyltransferase V (Vector Laboratories, Burlingame, USA). Protein bands were detected by chemiluminescense and X-ray film exposure (GE Healthcare).

Confocal microscopy

Cells were seeded on top of glass coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% bovine serum albumin (BSA) and incubated 1 h at room temperature with integrin-β1 antibody (Abcam). Alexa fluor 488 (Invitrogen, Life Technologies, NY, USA) was used as secondary antibody. Images were acquired on a Leica TCS SP5II confocal microscope (Leica Microsystems) using a Plan-Apochromat 63× oil objective. Images were processed using the LAS AF software (Leica Microsystems). Background noise was minimal when optimal gain/offset settings for the detectors were used. Digital images were optimized for contrast and brightness using Adobe Photoshop (Adobe Systems, San Jose, USA).

Adhesion assay

Cell adhesion assay was performed in 96-well plates, previously coated with fibronectin, type-I or type-IV collagen (all from Sigma-Aldrich) (5 μg/ml) overnight at 4°C. Plates were washed three times in PBS and non-specific-binding sites were blocked by adding 0.5% BSA (w/v) in PBS containing Pen/Strep for 2 h at 37°C. A total volume of 100 μl serum-free medium, containing 10^6 cells/ml, was plated in coated wells for 30 min, after which plates were washed with PBS to remove non-adherent cells, and the attached cells were fixed with acetone : methanol (1 : 1) for 10 min at 4°C. Cell adhesion was determined following the colorimetric method described by Busk *et al.* (47). The absorbance was measured at 570 nm with a microplate reader. The attachment of cells to wells coated with 1 mg/ml of poly-L-Lys and fixed with 4% paraformaldehyde before aspiration was defined as 100% of adhesion.

Imaging flow cytometry

To characterize the integrin-β1 and integrin-α5β1 distribution by imaging flow cytometry, 10^6 cells were seeded for 24 h, detached with Versene (GIBCO) and processed as live cells in suspension. Cells were incubated with integrin-β1 (Abcam) and integrin-α5β1 (Merck Millipore, Billerica, USA) antibodies for 1 h at room temperature. Alexa fluor 488 (Invitrogen) was used as the secondary antibody. Samples were analysed in the imaging flow cytometer (ImageStream®, Amnis, EDM Millipore), using a 488 nm excitation laser. All images were captured with the 40× objective (image pixel 0.5 μm^2) using the INSPIRE software (Amnis, EDM Millipore), acquiring at least 10 000 events.

Data were analysed using the IDEAS® software (Amnis, EDM Millipore, version 6.0.348) using only the single cell events. For the quantification of the fluorescence intensity of the labelled integrin-β1/integrin-α5β1 in the plasma membrane, a mask fitting the plasma membrane was built using the corresponding bright-field image, and fluorescence intensity was quantified within this region of interest.

Phenformin treatments

The effect of phenformin (Sigma-Aldrich) over respiration was evaluated by adding 5 mM phenformin before CCCP or oligomycin in oxygen consumption experiments described above. For confocal microscopy or imaging flow cytometry, 5 mM phenformin was added 1 h before fixation or cell harvesting, respectively.

Xenografts

All the procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, directive 86/609/EEC. N:NIH(s)II:nu/nu nude mice were subcutaneously injected in the dorsal flanks with 10^6 cells of WT or tRNAmut cybrids. Mouse weight and tumour width and length were measured with callipers every week. Excised tumours and lungs (to assess the presence of metastases) were fixed with 4% neutral buffered formalin and paraffin-embedded. The presence of lung metastases was evaluated by immunohistochemistry using anti-vimentin monoclonal antibody (DAKO, Glostrup, USA), and the streptavidin–biotin–horseradish peroxidase technique.

Statistical analysis

Statistical analysis was performed using GraphPad software (GraphPad Software Inc., La Jolla, USA), using Student's *t*-test or two-way ANOVA with Tukey *post hoc* test. Differences were considered statistically significant when $P < 0.05$ ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

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Conflict of Interest statement. None declared.

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3.2 – PAPER II: UGP2 Regulates ERK2-Driven EMT in Breast Epithelial Cells

UGP2 Regulates ERK2-Driven EMT in Breast Epithelial Cells

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Abstract

Most cancer-associated deaths are associated with metastasis. In epithelial cell-derived cancers, the formation of metastasis has been associated with transdifferentiation towards a mesenchymal phenotype, i.e. epithelial to mesenchymal transition (EMT). It is therefore crucial to have a detailed picture of the processes that govern EMT. Our interest has been directed to studying the metabolic reprogramming that occurs during EMT, a topic that is still widely unknown. To be able to perform a temporal characterization of the EMT process, we have created an inducible system where a non-tumorigenic mammary epithelial cell line (MCF10A) expresses a mutated form of ERK2 (D319N mutation), which was previously shown by our group to shift the ERK substrate preference and promote EMT. We have characterized the metabolism of these cells primed for EMT using metabolomics, revealing that, during EMT, cells undergo metabolic reprogramming, showing significant differences when compared with epithelial cells. Among these, cells undergoing EMT showed increasing levels of a metabolite, UDP-glucose, which comes from a non-classical and less explored glucose metabolic pathway. Moreover, these cells showed upregulation of the enzyme that catalyzes UDP-glucose production, UGP2, not only in the ERK2-driven system but also in other EMT models such as those driven by Ras, and TGF-β alone or plus TNF-α. Subsequent analyses showed that the expression of UGP2 is required for the EMT process in ERK2-driven system. UDP-glucose can integrate several pathways such as the production of UDP-glucuronate. This product of UDP-glucose, along with UDP-N-

acetylglucosamine (UDP-GlcNAc), the end-product of the hexosamine biosynthetic pathway, are also increased in cells undergoing EMT. Both UDP-glucuronate and UDP-GlcNAc are precursors of key molecules in migration and invasion of cells, including hyaluronan, which may be a key modulator of EMT. Taken together, our results show that UGP2 and the UDP-glucose pathway are associated with a EMT in a ERK2-D319N mammary model.

Introduction

Cancer cells undergo a metabolic reprogramming (Hanahan and Weinberg 2011). Metabolic re-wiring, which is considered today as a hallmark of cancer, was first explored by Warburg almost a century ago (Otto Warburg 1925) and arises from genetic alterations and from changes in oncogenic signaling, having an important role in tumorigenesis. Recent breakthroughs in cancer metabolism have shown that cancer cells have increased uptake of not only of glucose, but also amino acids such as glutamine, serine and glycine, while showing changes in the TCA cycle, oxidative phosphorylation and lipid metabolism, just to name a few (DeBerardinis and Thompson 2012; Vander Heiden, Cantley, and Thompson 2009). These studies revealed a robust picture of the metabolic needs of cell proliferation. However, less in known about the metabolic alterations associated with the formation of metastasis. Therefore, a key challenge is to understand the metabolic reprogramming of cancer cells during the acquisition of migratory and invasive features.

Epithelial to mesenchymal transition (EMT) is a process that occurs during development, in which epithelial cells lose their apico-basal polarity, reduce cell-cell contacts and acquire mesenchymal characteristics, namely fibroblast-like morphology and increased motility and invasion (M Angela Nieto 2013). This process has been shown to be recapitulated in some pathological conditions such as tissue fibrosis and cancer. In the latter, epithelial cell plasticity has also been associated with the acquisition of migratory and invasive properties and chemoresistance (Fischer et al. 2015; Nieto 2013). This cell plasticity is orchestrated by several transcription factors, like Snail, Slug, Zeb1 or Twist, that are responsible for the downregulation of epithelial proteins, such as E-cadherin, a key cell-cell adhesion protein, and upregulation of mesenchymal proteins (Thiery et al. 2009). One of the key features of EMT is the acquisition of migratory and invasive properties. It reflects changes in the expression of several cytoskeleton proteins such as vimentin, adhesion proteins such as E-cadherin and N-cadherin and secreted or shed proteins that modulate the extracellular microenvironment like fibronectin, collagen and metalloproteinases. Many of these molecules are glycosylated, a post-translation modification that regulates the localization and activity of proteins. Hyaluronan (or hyaluronic acid; HA) is one of the most abundant glycosaminoglycans of the extracellular matrix, organizing and maintaining its structural

integrity, that has been associated with tumor progression and aggressiveness (Toole 2004). This polymer is formed from products of the uridine diphosphate (UDP)-glucose and hexosamine biosynthesis pathways. By interacting with other proteins, such as cell membrane receptors, it can also function as a signaling molecule (Toole 2004). However, the role of HA in tumor progression is not clear because of the several functions of HA which depend on several factors such as its length, localization and its physical properties (Evanko and Wight 1999; Stern, Asari, and Sugahara 2006). On the other hand, the amount of HA and its length are also result of the balance of the enzymes that synthesize and degrade the molecule.

EMT can be triggered by several extracellular cues, such as TGF-β (Transforming Growth Factor-β), HGF (Hepatocyte Growth Factor), EGF (Epidermal Growth Factor), FGF (Fibroblast Growth factor), Notch/Jagged or phenomena like hypoxia (Thiery et al. 2009). ERK (Extracellular-signal Regulated Kinase), a Ras/MAPK (Mitogen-Activated Protein Kinase) pathway effector, has been shown to be at the cross-roads of several EMT inducers including TGF-β, HGF, EFG and FGF (Buonato and Lazzara 2014; Gonzalez and Medici 2014; Xie et al. 2004; Zhang et al. 2012). Previously, our group contributed with key findings on the ERK signaling that regulates EMT. We demonstrated that the D319N mutation in the CD (common docking) motif of active ERK2 promotes EMT (Shin, Dimitri et al. 2010) (Fig. 1). This point mutation does not affect the overall kinase activity but shifts the ERK substrate preference to DEF motif-containing substrates.

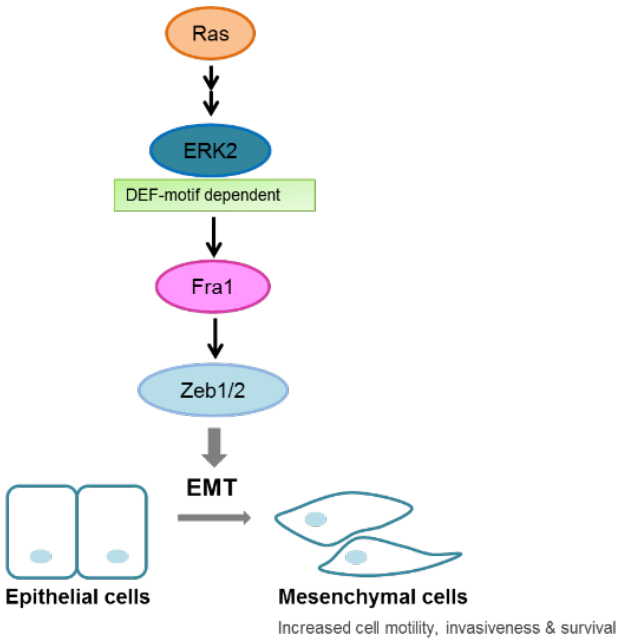


Fig. 1: ERK2 induces epithelial to mesenchymal transition via DEF motif-dependent signaling events (based on Shin and Blenis 2010).

In the current study, focused on understanding the metabolic requirements of EMT using our previously characterized ERK2-driven EMT model. Our goal was to define the metabolic landscape of the ERK2-DN cells as they undergo EMT, and then determine how metabolic pathways contribute to EMT. Our hypothesis is that cells reprogram their metabolism when they undergo EMT to support their migratory and invasive phenotype.

Material and Methods

Materials

All the materials used in this study are listed in the supplementary tables 1 to 9.

Cell lines and cell culture

MCF10A cells were acquired from ATCC. These cells and its derivatives were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) / Ham's F-12 50/50 Mix containing L-glutamine and 15 mM HEPES (Corning) supplemented with 5% horse serum, 20ng/mL EGF, 100ng/mL cholera toxin, 0.5µg /mL hydrocortisone and 10µg/mL insulin in a humidified incubator at 37ºC and 5% CO_2 and cultured according to Debnath et al. (Debnath, Muthuswamy, and Brugge 2003).

Stable cell lines overexpressing ERK2 were made by infecting MCF10A cells with tetracyclin (Tet) ON -inducible lentiviral plasmids either with a selectable marker for neomycin or puromycin. In the majority of the experiments, we used a construct containing a neomycin resistance gene, pINDUCER plasmid, containing the sequence for GFP (control) or for the rat HA-tagged ERK2 – wild-type (WT) or with an Y261A or D319N mutation. The rat HA-tagged ERK2 with a Y261A or D319N mutation was also cloned to an pTRIPZ plasmid (contains a puromycin resistance gene). These cell lines were named GFP (pINDUCER control), empty vector (pTRIPZ control), ERK2-WT, ERK2-YA and ERK2-DN cells.

Stable cell lines with UGP2 knock-down were obtained by infecting GFP control or ERK2-DN cells with the pRRL-Lenti-miRE-Ren713 plasmid containing shRNAmir against UGP2, according to the protocol on the “shRNAmir system” section. The expression was induced with 0.5µg/mL of doxycycline (DOX) (Calbiochem).

Cell lines overexpressing UGP2 isoforms were made by infecting MCF10A cells with the pCW57.1 plasmid containing the sequences of UGP2 isoform 1 or UGP2 isoform 2 (see “Gateway cloning” section for the details on the plasmids production). The expression was induced with 0.5µg/mL of DOX.

Cell lines expressing shRNA sequences against the HAS2 gene were made by infecting GFP control or ERK2-DN cells with the pLKO.1 plasmid with the shRNA sequences.

Cells containing the pINDUCER construct (ERK2 and RasV12 expression) were maintained in media with 300µg/mL G418 (the selection agent in eukaryotic cells containing the neomycin

phosphotransferase gene). On the other hand, the cells containing pTRIPZ (ERK2 expression), pRRL-Lenti-miRE-Ren713 (shRNAmir), pCW57.1 (UGP2 expression) or pLKO.1 (shHAS2) constructs were cultured in media with 2µg/mL puromycin.

Assay media used in the migration and invasion assays using transwells is the same media used for MCF10A cells but without EGF or insulin and with only 2% horse serum.

HEK-293T, used to generate virus, were obtained from GenHunter (Q401) and cultured in DMEM, high glucose (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich).

All the cells lines were not kept in culture for more than 3 weeks.

AZD6244 treatment

GFP control, ERK2 WT and DN cells were treated with DOX for 7 days and on day 5 were plated on 6-well plates. On day 7, cells were treated with 1µM or 2µM of AZD6244 or the vehicle control dimethyl sulfoxide (DMSO) for 4h. After the incubation period, lysates were collected and run as described in the "Western Blotting" section.

shRNAmir system

shRNAmir technology was used to knock-down the UGP2 gene (Dickins et al. 2005; Stegmeier et al. 2005; Zeng, Cai, and Cullen 2005; Zeng, Wagner, and Cullen 2002). We used the protocol established by Dow, Premsrirut and Zuberet *et al.* (Dow et al. 2012)(Dow, Premsrirut and Zuberet et al. 2012) and the pRRL-Lenti-miRE-Ren713 plasmid (Fellmann et al. 2013).

The 97mer were designed using splashRNA.mskcc.org and ordered through Invitrogen (table 9). We designed 10 oligonucleotides for the UGP2 gene. The PCR cloning of the shRNAs was performed using the high-fidelity polymerase "Platinum™ Pfx DNA Polymerase" (Invitrogen) and the PCR product was run on an agarose gel. After confirming the presence of a band in the expected size (131 base pairs), the remaining product was isolated using the "E.Z.N.A.® Cycle Pure Kit (V-spin)" (Omega). The ligation reaction was performed using "T4 DNA Ligase" (New England BioLabs) and each previously digested fragment was ligated to the Tet-ON inducible plasmid (similarly digested) pRRL-Lenti-miRE-Ren713 (Fellmann et al. 2013). After bacterial transformation, 4 colonies per ligation reaction product were purified using "PureLink™ Quick Plasmid Miniprep Kit" (Invitrogen) and sequenced. A product with a correct sequence was selected per sequence of shRNA and transfected into HEK-293T cells to produce virus to subsequently infect MCF10A + ERK2 DN and control cells. The knock-down efficiency of each

shRNA was assessed by Western blotting and from the total of 10 sequences tested, the 2 constructs that achieved higher knock-down were selected. Cells infected with the plasmid pRRL-Lenti-miRE-Ren713 targeting *Renilla* luciferase 713 (shRen) were used as control.

shRNA

A total of 8 sequences were tested and the 3 constructs that achieved higher knock-down were selected. shRNA constructs were from the RNAi Consortium (TRC) at the Broad Institute: shGFP (TRCN0000072181), shHAS2 #1 (TRCN0000045393, targeting CDS) shHAS2 #6 (TRCN0000045396, targeting CDS) and shHAS2 #8 (TRCN0000418983, targeting CDS) in a pLKO.1 backbone.

Gateway cloning

The Gateway technology (Hartley, Temple, and Brasch 2000) was used to produce the expression plasmids of both isoforms of UGP2 with the goal of overexpressing them in MCF10A cells. Two constructs containing the sequence of the open reading frame of both UGP2 isoforms was commercially available: the attB expression clone pOTB7 that has the sequence for UGP2 isoform 2 and the entry clone pENTR223 that contains the sequence for the isoform 1 of UGP2. First, the entry clone for pOTB7 UGP2 was generated by performing the BP recombination reaction (attB × attP recombination, where "attB" stands for recombination attachment sites in bacteria and "attP" stands for such sites in phage) using the "Gateway™ BP Clonase™ II Enzyme mix" (Invitrogen) and the pDONR vector (containing attP sites). A positive control vector was used to monitor the efficiency of the reaction. The products of this reaction were amplified by transforming competent *E. coli* bacteria with 2µL of each product. Independent colonies for each clone were isolated and the plasmid DNA was purified using "PureLink™ Quick Plasmid Miniprep Kit" (Invitrogen) according to product's manual. To confirm the sequence, the expression clones were sequenced. Subsequently, both entry clones, the one originated from pOTB.7 and the pENTR223 – together with the control pENTRY-GFP (Addgene; 15301) were transferred into the pCW57.1 lentiviral TetON-inducible puromycin-resistant destination vector (Addgene; 41393) by LR recombination reaction (attL × attR recombination, where "L" = "left" and "R" = "right"). This was achieved using the "Gateway™ LR Clonase™ II Enzyme mix" (Life Technologies; 11791-100). The products of this reaction – the expression clones - were amplified by transforming competent *E. coli* bacteria with 2µL of each product. Independent colonies for each construct were isolated and the plasmid DNA was purified using the same miniprep kit,

according to product's manual. To confirm the sequence, the expression clones were sequenced. Virus containing the expression plasmids were produced and used to infect MCF10A cells.

Plasmids and generation of stable cells

HEK-293T cells were used to generate lentivirus. Cells were plated in 6-well plates coated with a solution of 0.2% gelatin (in 1x phosphate-buffered saline [PBS]) in order to reach approximately 70-80% density on the day of the transfection. Then, plasmids were transfected into HEK-293T cells using Lipofectamine® 2000 Transfection Reagent (Invitrogen by ThermoFisher Scientific; 11668019) with the lentiviral expression plasmids for packaging (Δ8.9) and envelope (VSV-G), and medium was changed the next day, using media with 20% FBS. When producing virus carrying the shRNAmir, a shRNA targeting Pasha was co-transfected to enhance virus titer (Chang et al. 2013). After 48h, virus-containing supernatants were collected and new medium with 20% FBS was added. Additional virus-containing supernatants were collected after another 48h and the total supernatant was filtered using 0.45µm filters.

Cells were plated in order to reach about 50% density on the following day. Cells were infected with virus-containing supernatants in the presence of a serum-containing medium (ratio 1:1) supplemented with 8 µg/mL polybrene. Virus-containing medium was removed after 24h and cells were grown in serum-containing medium for another day. Cells were then treated with puromycin (2µg/mL) or G418 (300µg/mL) for selection. A "kill plate" with non-infected cells was used to monitor the antibiotic efficacy (the selection was only considered completed when there are no live cells in the "kill plate"). The knockdown efficiency was confirmed by immunoblot analysis.

EMT models

MCF10A cells infected with the TetON inducible lentiviral pINDUCER ERK2-WT, ERK2-YA and ERK2-DN constructs or infected with the TetON inducible lentiviral pTRIPZ ERK2-YA and ERK2-DN constructs were plated at a cell density of approximately 1.7×10^4 cells/cm^2 on day -1. After 24h (on day 0), the cell media was replenished and treatment with DOX at a final concentration of 0.5µg/mL was started and performed every 24h. The morphology of cells was monitored every day using a bright field microscope. Cells were trypsinized and re-plated every other day (days 1, 3, 5 and 7) and media was replenished (containing fresh DOX) on the day in between. On days 0, 1, 3, 5, 7 and 9 cells were at an approximate density of 80% and data was collected. The same protocol was used for MCF10A expressing pINDUCER HRAS G12V.

MCF10A cells were plated at a cell density of 1.7 x 10^4 cells/cm^2 and treated with 5ng/mL TGF-β (PeproTech) or 5ng/mL TGF-β and 5ng/mL TNF-α (Tumor Necrosis Factor-α) (PeproTech) on day 0. The morphology of cells was monitored every day using a bright field microscope. Cells were trypsinized and re-plated every other day (days 2, 4, 6, 8 and 10) and media was replenished (containing TGF-β or TGF-β and TNF-α) on the day in between. On days 0, 2, 4, 6, 8 and 10 cells were at an approximate density of 80% and data was collected.

Migration and invasion

Migration - wound healing assay

Cells were plated in 12-well plates in order to reach a confluent monolayer after 48h, on timepoint day. The cell monolayer of each well was scraped in a straight line to create a "scratch" with a p200 pipet tip. It was important that each scratch has a similar width. In In order to remove the debris and smooth the edge of the scratch, cells were washed once with growth medium and then fresh media was added. The time-lapse microscopy was performed using a Nikon Eclipse Ti-E microscope (Nikon) that has the NIS-Elements software (Nikon). The tissue plate was placed in the pre-heated cell incubator at 37°C and the fields to be monitored over time selected. Pictures were acquired every 5min. for 12h with an amplification of 10x. The area of the wound in (μm^2) was measured in several timepoints. The biological replicates were averaged and normalized to the area of the wound on t=0 of each condition. Migration was defined as the capacity to migrate into the wound and measured as the percentage of wound closure through time.

Migration assay using transwells

Before plating the cells, the transwells "Transparent PEM Membrane 24-well 8.0 µm pore size" (VWR) were placed on the wells of a 24-well plate and the top of the membrane was blocked for 1h at 37ºC using assay media. Cells (5×10^4 in 250µL of assay media) were added to the top chamber of the transwells and 600 µL of Assay media + 5ng/mL EGF was added to the bottom chamber of the well. The cells were allowed to migrate through the membrane for 6h or 12h (shUGP2 and shHAS2). After the desired time, the viability of cells was confirmed using a microscope, the cells from the top side of the membrane were removed and the cells that migrated the lower surface of the membrane were fixed using 100% ethanol for 10min at room temperature (RT). Cells were then stained using a solution of 0.2% crystal violet (in 2% ethanol) for 10min at RT. Migration was accessed using an inverted microscope and pictures with a 4x objective were taken.

Invasion assay using transwells

The invasion assay using transwells was performed as described in the migration assay but using transwells coated with Basement Membrane Extract (BME) prepared from "Cultrex® 5x BME Solution" (Trevigen) in filtered ddH20 or available commercially precoated transwells "Matrigel® Invasion Chamber 24-well Plate 8.0 Micron" (Corning). The transwells either coated with BME or Matrigel® were rehydrated before use for 2h at 37ºC with assay media. In the bottom chamber of the well, 600µL of Assay media + 20ng/mL EGF was added and the cells were allowed to invade for 22h or 24h (shUGP2).

Immunofluorescence

Cells were plated in coverslips placed into 6-well plates. On the day of the timepoint, cells were washed with 1x PBS, fixed using 4% formaldehyde (in 1xPBS) for 10min at RT. Cells were rinsed four times in 1x PBS and blocked for 10min with a solution of 3% bovine serum albumin (in 1x PBS). The primary antibody for E-cadherin (BD Transduction Laboratories, 610181) was diluted 1:50 in the blocking solution and incubated for 2h at RT. Negative controls, where the primary antibody was not added, were included in the experiment in order to control for non-specific staining of the secondary antibody. After rinsing the coverslips four times in 1x PBS, the cells were incubated with the secondary antibody conjugated to a fluorochrome "Alexa Fluor 488 goat anti-mouse IgG (H+L)" (Thermo Fisher Scientific) diluted 1:1000 in blocking solution for 2h at RT. After rinsing the cells three times with 1x PBS, the nucleus was counterstained using "Hoechst 33258, Pentahydrate (bis-Benzimide)" (Thermo Fisher Scientific) and then washed again once. Cells were imaged in a Nikon Eclipse Ti-E microscope (Nikon).

HA binding protein staining

After treatment of MCF10A cells with TFG-β for 4 days, cells were washed once with 1x PBS and fixed in a solution of 4% formaldehyde (in 1x PBS) for 10min at RT. Then, cells were rinsed twice with 1x PBS and blocked with 1% BSA (in 1x PBS) for 1h. The incubation with "Hyaluronic Acid Binding Protein-Biotin bovine" (Sigma) was done using a final concentration of 10µg/mL o/n at 4ºC. After the incubation, cells were rinsed three times with 1x PBS and incubated with the secondary antibody "Alexa Fluor® 568 streptavidin" at a dilution of 1:1000 (in blocking solution) for 1h at RT. After rinsing the cells three times with 1x PBS, the nucleus was counterstained using "Hoechst 33258, Pentahydrate (bis-Benzimide)" (Thermo Fisher Scientific) and then washed again once. Cells were imaged in a Nikon Eclipse Ti-E microscope (Nikon).

Glucose uptake

Glucose uptake was measured using the "Glucose Uptake Colorimetric Assay Kit" (Biovision; K676), according to the manufacturer's instructions. This assay uses an analogue of glucose, 2-deoxy-D-glucose (2-DG) that is taken up by cells, through glucose transporters and metabolized to 2-DG-6-phosphate (2-DG6P). The latter cannot be further metabolized and thus accumulates in the cells. The accumulated 2-DG6P is directly proportional to 2-DG uptake by cells. After some enzymatic reactions, the end-product is measured by absorbance. Briefly, cells were washed with 1x PBS and then starved for 1h with DMEM media without glucose and pyruvate + 10% dialyzed FBS, that proceeded the incubation with 2-DG for 40min. (except in control cells which were incubated with media with normal glucose). After washing the cells to remove the remaining extracellular 2-DG, cells were lysed and added to the plate, diluted in the reaction mix. A standard with known glucose concentrations was added to the plate to extrapolate the glucose uptake from the samples. The absorbance at 412nm was measured in the plate reader Envision 2014 Multimode Plate Reader (PerkinElmer) after 30min at 37ºC. Glucose uptake was calculated using the standard curve and normalized to cell number.

Lactate secretion

Lactate concentration in the cell media was measured using a BioProfile FLEX Analyzer (Nova Biomedical). Lactate secretion was determined by subtracting the values for lactate in the experimental conditions from the values in the respective control media (no cells) and normalized to protein concentration - µg/µL of protein – (see "Western Blotting" section). The average and standard deviation were calculated for 3 biological replicates.

Metabolites' extraction and LC-MS/MS

Polar metabolites were extracted using a solution of 80% methanol (for HPLC gradient grade ≥99.9%) according to Yuan et al. (Yuan et al. 2012). Briefly, cells seeded in 15cm plates were washed once with 1x PBS and cell culture media was replenished 2h before metabolite extraction. The cell culture media from each cell plate was aspirated completely and cells were washed once with 1x PBS. The plates were put on dry ice and a cooled to -80ºC solution of 80% methanol (vol/vol, in ddH_2O) was added to the plate. Cells were incubated for 15min at -80ºC and scraped off the plate on dry ice using a cell lifter. The cell lysate/methanol mixture was centrifuged at 14000g for 5min at 4ºC. The metabolites-containing supernatant was transferred to a new tube and the pellet was washed with 80% methanol and pellet. The metabolites-containing supernatant was dried using a SpeedVac (no heat) and the metabolites were stored at -80ºC until further use.

Before the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the dried pellets were resuspended in 20µL of LS/MS-grade water, centrifuged for 2min. at 1000rpm and the supernatant was transferred to LS-MS/MS system tubes. The quantitative steady-state metabolomics profiling was performed using LC-MS/MS by the Beth Israel Deaconess Medical Center Mass Spectrometry Core Facility (Boston, USA) headed by John M. Asara, according to Yuan *et al.* (Yuan et al. 2012).

Metabolomics data analysis

The metabolomics data obtained was the peak area integrated total ion chromatogram (TIC) values. Samples were normalized to protein concentration – µg/µL - (see "Western Blotting" section) on a duplicate set of cells treated identically to the experimental cells and the average of 3 biological replicates was calculated.

The heatmap was created using MeV Multi Experiment 4.9 data analysis and visualization software. Metabolomics data (treated as mentioned previously) was input and the following analysis was performed: hierarchical clustering, using the average linkage as linkage method and the distance metric the Pearson correlation.

Gene expression analysis using real-time PCR (qPCR)

RNA was isolated using RNeasy Mini Kit (QIAGEN) or "PureLink™ RNA Mini Kit" (Ambion) according to the product's manual. One µg of total RNA was treated with "DNase I Amplification Grade" (Sigma Aldrich) and was reverse transcribed by using "iScript™ cDNA Synthesis Kit" (Bio Rad) according to the manufacturer's guidelines. The resulting cDNA was diluted in nuclease-free water (1:5). qPCR was performed with gene specific primers at 50ºC for 2min. and 95°C for 10 sec (hold stage), 40 cycles at 95ºC for 15sec and 60°C for 1min. (PCR stage) and 95ºC for 15sec, 60ºC for 1min. and 95ºC 15sec ((melt curve stage) in 10µl reaction mix containing 2µl cDNA (20ng), 1µl mix of primers reverse and forward and 5µl of "SYBR™ Green PCR Master Mix" (Life Technologies) using a "Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System" (ThermoFisher Scientific) thermocycler. Actin and TATA box binding protein (TBP) were used as an endogenous control. After assuring that the efficiency of the amplification between the target gene and endogenous control were similar, the quantification was performed using the ΔΔCt method (Schmittgen and Livak 2008). See Table 8 for the primer sequences used for qPCR analysis.

Luciferase promoter activity assay

The construct of the *Renilla* luciferase gene under the promoter of UGP2 and the *Cypridina* TK control promoter construct were purchased to SwitchGear Genomics. Cells plated on a 6-well plate were co-transfected with 2µg of the construct containing the *Renilla* luciferase gene under the promoter of UGP2 (or the empty vector control under a minimal promoter) and 1µg of control *Cypridina* construct – the co-transfection control- using "X-tremeGENE™ HP DNA Transfection Reagent" (Roche). 48h after transfection, the activity of luciferase was measured using the "LightSwitch™ Dual Assay Kit" (Active Motif), according to the manufacturer's protocol. The absorbance was measured and the *Renilla* luciferase activity was normalized by *Cypridina* luciferase activity.

Lambda Phosphatase treatment

Protein (30µg) was resuspended in a mixture of 20 units of phosphatase "Lambda Protein Phosphatase (Lambda PP)" (New England BioLabs) per µg of protein, in the phosphatase buffer and $MnCl_2$ provided with the enzyme. The tubes were vortexed and incubated for 1h at 30ºC in a water bath. Control samples were treated identically and simultaneously, but without any phosphatase. According to the manufacture, 100 units of Lambda PP remove ~ 100% of phosphates (0.5nmol) in phosphorylated myelin basic protein (18.5 kDa) in 30min. in a 50µL reaction. The concentration of phosphorylated myelin basic protein is 10µM with respect to phosphate, as described by the manufacturers. After the incubation, the Western blotting loading buffer (see "Western Blotting" section) was added to the lysates, followed by boiling at 95ºC and the Western blotting protocol was continued according to the "Western Blotting" section.

Western Blotting

Cells cultured in plates were washed once with 1x PBS before lysis. Cells were lysed and protein precipitated in a solution of 10% trichloroacetic acid (in 10mM Tris-HCL pH8.0, 25mM NH_4OAc and 1mM Na_2EDTA) and by scrapping cells off the plate using a cell lifter. Cell lysates were centrifuged at 13200 rpm for 10min. The supernatant was aspirated and the pellet was resuspended in 3% SDS buffer (in 0.1M Tris-HCL, pH 11.0) and boiled at 95ºC for 10 min.

Cell lysates were quantified using an adapted Lowry method provided by the DC™ Protein Assay (Bio-Rad). Samples were normalized to 2.5µg/µL in a 2% SDS, 10% glycerol, bromophenol blue, 0.1% β-mercaptoethanol (in 0.2M Tris-Cl, pH 6.8) loading buffer and boiled for 5 min. A total of 25µg of protein were loaded into each well and electrophoresed by SDS-PAGE using 10%

acrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane – "Amersham Protran 0.45 NC" (GE Healthcare) - in a 10% methanol glycine-based transfer buffer.

Immunoblotting with the primary antibody (see table 1) diluted in 1x Tris-buffered saline with Tween (TBS-T) (25mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5% Tween® 20) was performed after blocking the membrane in "Odyssey® Blocking Buffer (TBS)" (LI-COR) (in 1x TBS) in a rocking shaker o/n at 4ºC. "IRDye® 800CW" or "IRDye® 680RD" secondary antibodies (LI-COR Biosystems) were incubated at a 1:20000 dilution in TBS-T for 1h at RT. Membranes were washed after the primary and secondary antibodies with TBS-T. Immunoblot signals were detected by Odyssey® CLx Imaging System (LI-COR Biosciences).

Slug antibody and the corresponding secondary antibody - "ECL anti-Rabbit IgG, HRP-linked, whole molecule, from donkey" (GE Healthcare - Life Sciences) - were diluted 1:5000 in a solution of 5% nonfat dried milk (in TBS-T). Slug was developed using enhanced chemiluminescence (ECL). Immunoblot images are representative of at least two independent experiments.

In the case of the detection of glycoproteins using lectins, the Western blot was performed according the above described, except 5 µg of protein was loaded in an 8% acrylamide gel. Besides, the membranes were blocked with 4% BSA (in PBS with 0.05% -Tween® [PBS-T]). The lectins were diluted in a solution of 1% BSA (in PBS-T) and incubated for 45min at RT. After washing the membranes three times for 10min, they were incubated with the avidin/biotin technology reagents from the "Vectastain® Elite® ABC-HRP Kit" (Vector Laboratories) for 45min at RT. After washing the membranes three times for 10min, they were developed using ECL.

Vinculin and actin expression were used as loading controls.

Glycogen – PAS staining

Detects polysaccharides (such as glycogen) and glycoconjugates. Based upon the reactivity of free aldehyde groups within carbohydrates with the Schiff reagent to form a bright red magenta end product.

The protocol adapted from Shen *et al.* (Shen et al. 2010) and the reagents used were part of the "Periodic Acid-Schiff (PAS) Kit" (Sigma). Briefly, cells were plated in coverslips placed into 6-well plates. On the timepoint day, medium was removed, cells washed with 1x PBS and fixed with Carnoy's fixative (3 parts ethanol and 1 part glacial acetic acid) for 1h at RT. The cells were rinsed with absolute alcohol and 66% alcohol once for 2 min, followed by rinsing with deionized water three times for 30sec. The cells were treated with periodic acid solution for 10 min and then

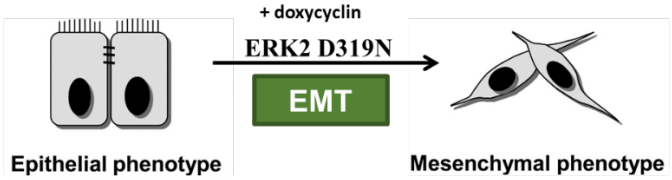
rinsed with deionized water three times for 30sec. Then the cells were treated with Shiff reagent for 20min in the hood, followed by 5min. with running tap water. After air-drying, the stained cells were covered with a glass slides using pure glycerol as mounting medium.

Results

Establishment of the doxycycline-induced ERK2-D319N EMT model

In order to study the metabolic requirements of EMT, we generated a doxycycline (DOX)-inducible ERK2-driven EMT model. In this model, the non-tumorigenic mammary epithelial cell line MCF10A expresses a mutated form of ERK2, ERK2-D319N (ERK2-DN cells). This mutation – an aspartic acid (D) to asparagine (N) – is located in the CD motif of ERK2, shifting the ERK substrate preference to DEF motif-containing substrates and had previously been described by our group as capable of inducing EMT (Shin and Dimitri et al. 2010). On the other hand, the ERK2-Y261A mutation affects the DEF-motif binding pocket, resulting in ERK2 preferentially signaling to D-motif substrates. Cells expressing the ERK2-DBP mutant (ERK2-YA cells) do not exhibit morphological changes and will be used as a control in the first part of this paper. The overexpression of the WT form of ERK2 (ERK2-WT cells) results in a milder EMT phenotype (Shin, Dimitri et al. 2010). As a control, we used MCF10A cells infected with an empty or GFP vector (vector or GFP cells). We optimized a DOX-inducible system (3rd generation tet-HA-ERK2) that provides a temporal characterization of EMT (Fig. 2). All the ERK2 constructs - ERK2-WT, ERK2-YA and ERK2-DN - are HA-tagged. Compared to control vector cells and ERK2-YA cells, within a few days after DOX treatment, ERK2-DN cells changed their epithelial cellular morphology, became more elongated and fibroblast-like and exhibited alterations in the expression of epithelial (E-cadherin) and mesenchymal-associated proteins (fibronectin, N-cadherin and Zeb1) (Fig. 3). Specifically, ERK2-DN cells downregulate E-cadherin and upregulate fibronectin, N-cadherin and Zeb1 over time (Fig. 3 and Shin, Dimitri et al. 2010). The downregulation of E-cadherin is also evident by immunofluorescence staining, while in vector and ERK2-YA cells, E-cadherin is localized at the membrane (Fig. 3 and Shin, Dimitri et al. 2010).

A

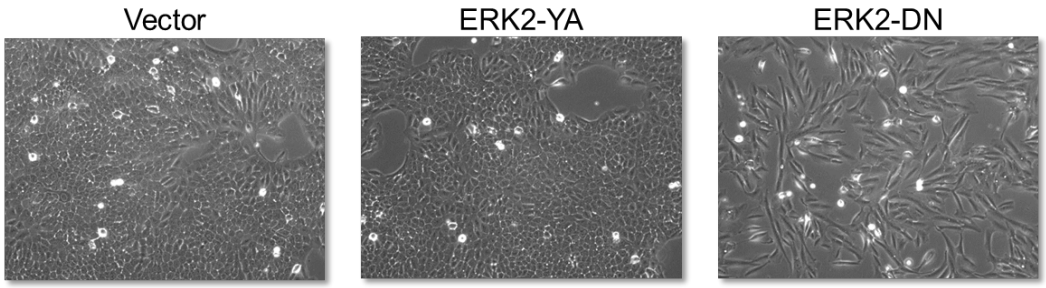


B

MCF10A + **Vector / GFP** control
ERK2 **WT**
ERK2 **DN** mutation (D319N mutation in the common docking site)
ERK2 **YA** mutation (Y261A mutation affects the DEF-motif binding pocket)

Fig. 2: ERK2-driven EMT model (A) and cell systems used (B).

A



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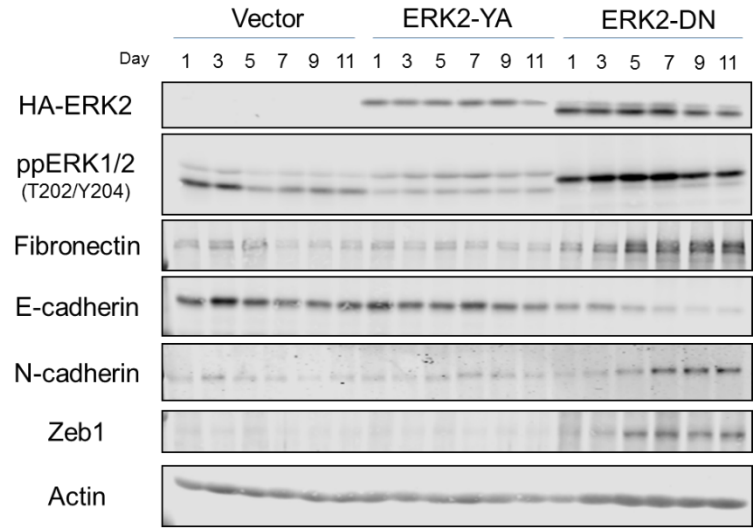


C

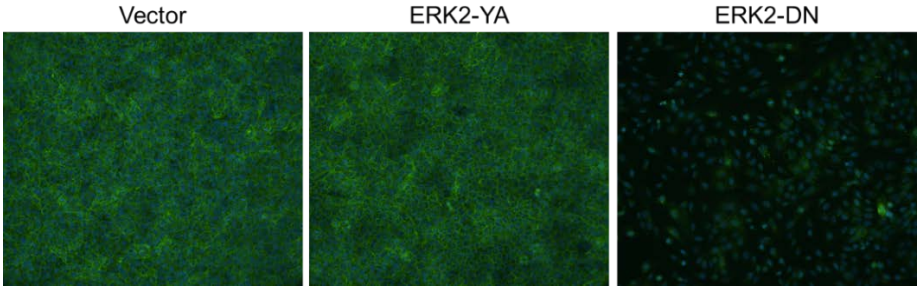


Fig. 3: Morphology after 11 days of DOX treatment (A), expression of the EMT markers during the time-course DOX treatment (B) and immunofluorescence staining for E-cadherin (green) after 9 days of DOX treatment (C) in ERK2 -YA and -DN mutants compared to the vector control. The phase contrast images were taken with a 10x objective. The immunofluorescence images were taken with a 20x objective and the nucleus was counterstained with Hoechst (blue). The experiment was repeated at least 3 times.

One of the most important features of EMT is increased migration. Consequently, we performed a wound healing assay in order to assess the ability of cells to migrate and close a “scratch” or wound in the cell monolayer. We also analyzed the migratory and invasive capacity of the cells

through a porous membrane in non-coated transwells (migration) or in transwells coated with basement membrane extract (BME), a commercially available matrix (invasion). As expected, ERK2-DN cells displayed enhanced migratory and invasive capacities when compared to control cells or ERK2-YA, as measured by the percentage of wound closure over time (Fig. 4A) and by staining the cells that have migrated through the porous membrane (Fig. 4B).

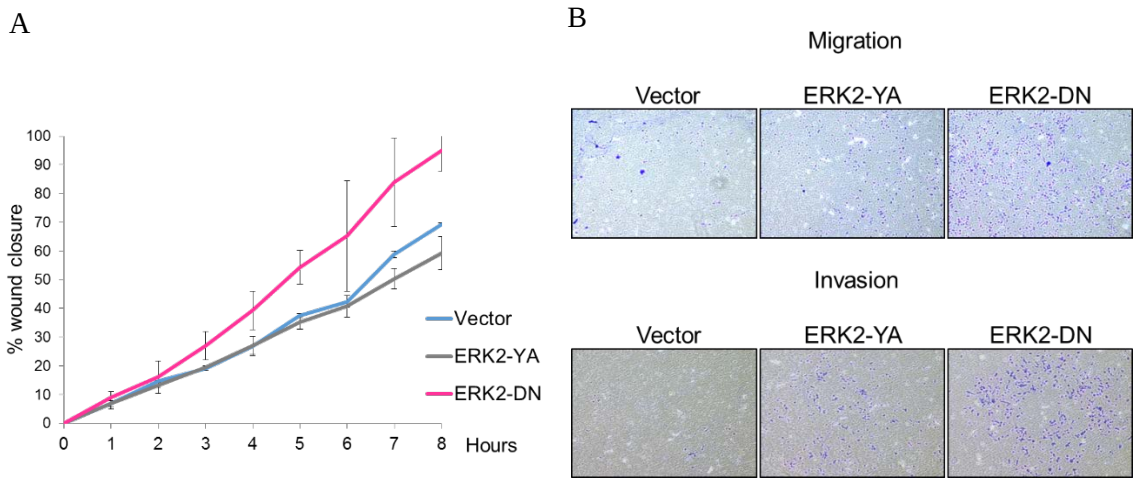


Fig. 4: Wound healing assay (A) and migration and invasion of cells in non-coated or BME-coated transwells (B) in ERK2 YA and DN mutants compared to the vector control during the time-course DOX treatment (wound-healing) or after 7 days of DOX treatment (migration and invasion in transwells). Both experiments were repeated at least 3 times. Data from the graph are represented as mean ± SD.

Glucose consumption and lactate secretion of ERK2-driven EMT

Having established a suitable model, our goal was to identify the metabolic alterations associated with ERK2-driven EMT. Firstly, we measured the glucose consumption using a 2-DG assay in cells undergoing EMT. This assay uses an analogue of glucose, 2-DG, that is taken up by cells, through glucose transporters and quantifies an intracellular product that accumulates in the cells. After 9 days of DOX treatment, ERK2-DN cells consume less glucose than vector control and ERK2-YA cells (Fig. 5). Accordingly, after analyzing the protein expression of one of the glucose transporters, we saw that GLUT1 is downregulated in ERK2-DN after DOX treatment (Fig. 6). Moreover, we assessed lactate production by quantifying this metabolite in the cell media. We concluded that ERK2-DN cells secrete less lactate to the cell media (Fig. 5).

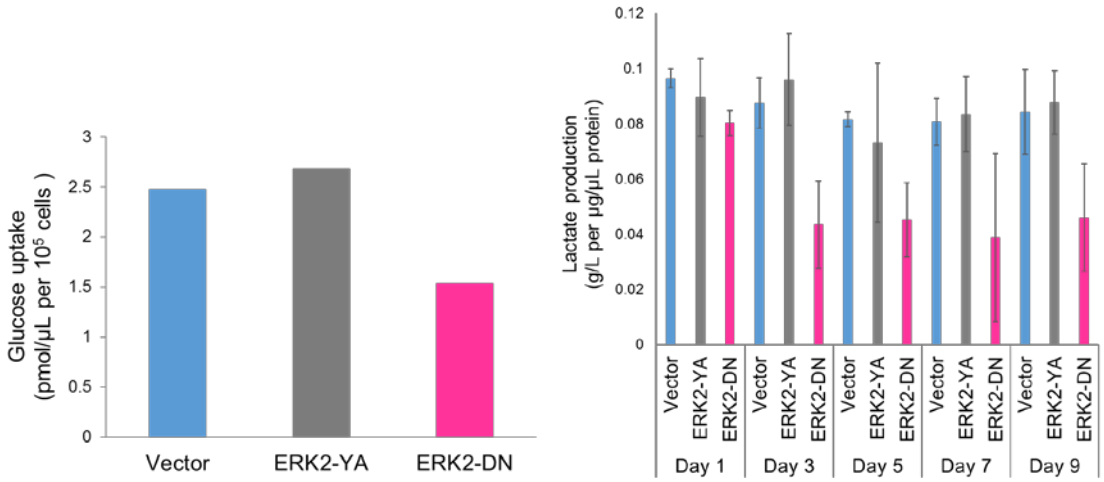


Fig 5: Glucose uptake and lactate secretion in ERK2 -YA and -DN mutants compared to the vector control. The glucose uptake was measured using 2-DG assay after 9 days of DOX treatment and the lactate production was determined in the cell media using a BioProfile FLEX Analyzer (Nova Biomedical) during the time-course DOX treatment. Data of lactate production are represented as mean ± SD.

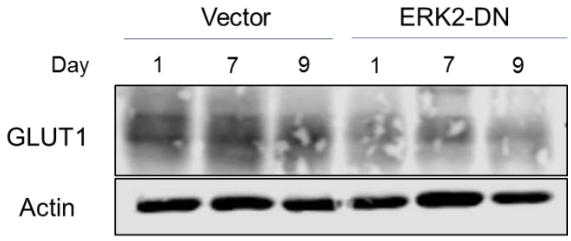


Fig. 6: Protein expression of GLUT1 in ERK2-DN cells compared to vector control after 1, 7 and 9 days of DOX treatment.

Metabolic reprogramming and increased UDP-glucose levels during EMT

In order to define the metabolic effectors that regulate EMT in this model, we utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) to perform a quantitative metabolomics profiling analysis and determine the metabolic program associated with the transition. During the experiment, the endogenous polar metabolites were extracted from ERK2 -DN and -YA cells (in addition to the empty vector control cells) in several time-points during a 9-day experiment. The data was normalized to protein concentration. By analyzing the relative abundance of the metabolites of DN cells and YA cells (normalized to vector control) over time, we concluded that there is dramatic metabolic reprogramming in these cells during the EMT (Fig. 7A). Notwithstanding the low glucose consumption, we were intrigued by the reduced lactate

production during EMT and questioned the fate of glucose in ERK2-DN cells. Interestingly, one of the metabolites whose levels were markedly increased over time in DN cells (and not in YA or vector control cells) was UDP-glucose (Fig. 7B). UDP-glucose is an intermediate metabolite in several pathways of the carbohydrate metabolism that can be synthetized from glucose after its conversion into glucose 6-phophate and glucose-1-phosphate.

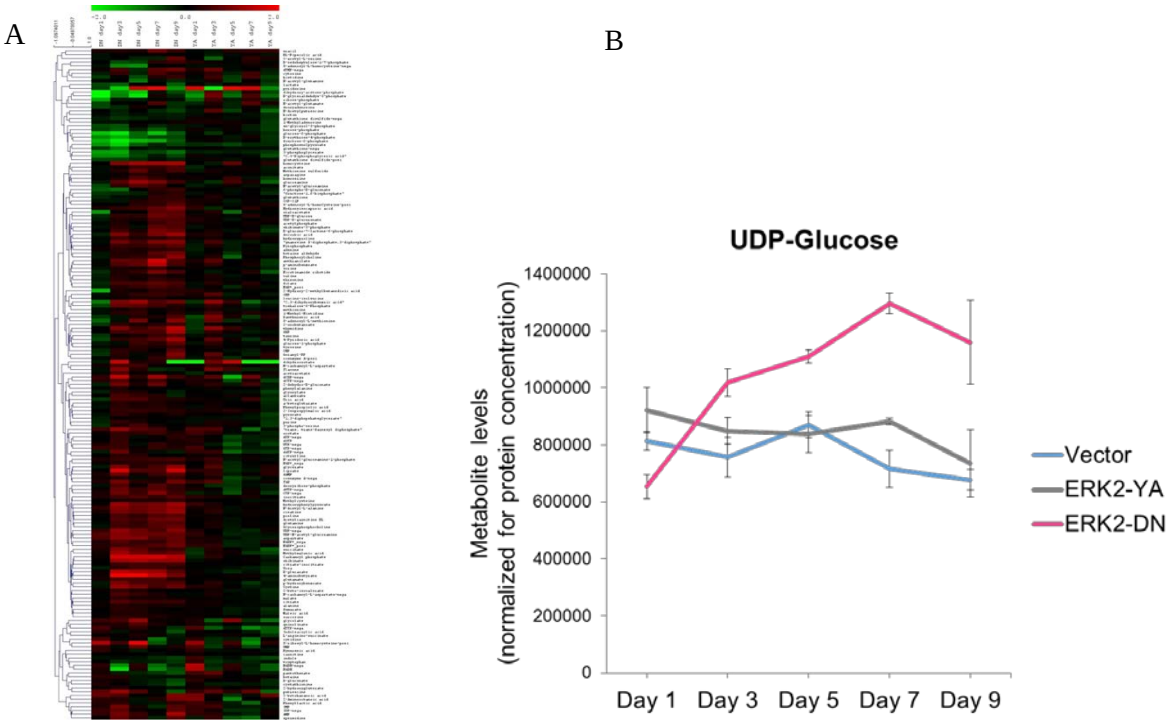
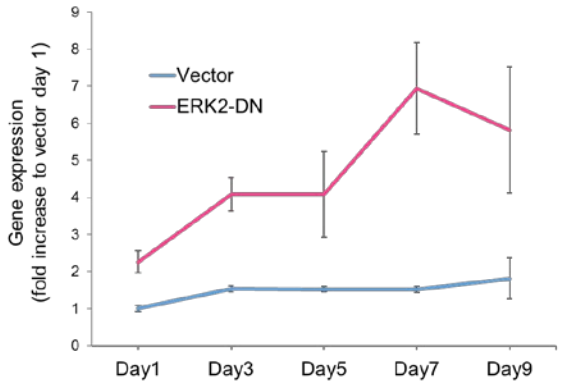


Fig. 7: Metabolic profiling (A) and UDP-glucose levels (B) in ERK2 -YA and -DN mutants compared to the vector control during the time-course DOX treatment. The graphic represents the quantification of integrated peak areas TIC values. The experiment was repeated 3 times. Data are represented as mean ± SD.

UGP2 is upregulated during EMT

To identify the molecular mechanism behind the increased UDP-glucose levels during EMT, we analyzed the expression level of the enzyme responsible for UDP-glucose production, UDP-glucose pyrophosphorylase 2 (UGP2). This enzyme uses glucose-1-phosphate and UTP as substrate to produce UDP-glucose, in a reversible reaction (Fuhring *et al*. 2015). Consistent with the increased UDP-glucose production, UGP2 expression was upregulated at both the mRNA and protein level in DN cells (Fig. 8). Additionally, ERK2-WT overexpressing cells also upregulate UGP2, although to a lesser extent in accordance with their mild EMT (Fig. 8B).

A



B

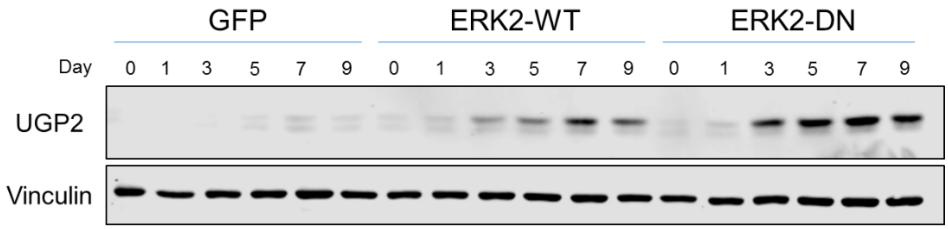


Fig. 8: Expression of UGP2 at the mRNA (A) and protein (B) level in ERK2 WT and DN cells compared to the vector control during the time-course experiment. Both experiments were repeated at least 3 times. mRNA data are represented as mean ± SD.

Expression of UGP2 is increased in different EMT models

Since UGP2 is the enzyme responsible for the production of UDP-glucose, we further explored the expression of this enzyme during EMT, questioning whether UGP2 is also upregulated in other EMT models. We analyzed the expression of UGP2 in MCF10A either treated with TGF-β or TGF-β plus TNF-α, or with DOX-inducible overexpression of HRAS-G12V (Thiery et al. 2009). Consistent with the results from our ERK2-driven EMT model, UGP2 was upregulated in the mesenchymal cells from these systems (Fig. 9).

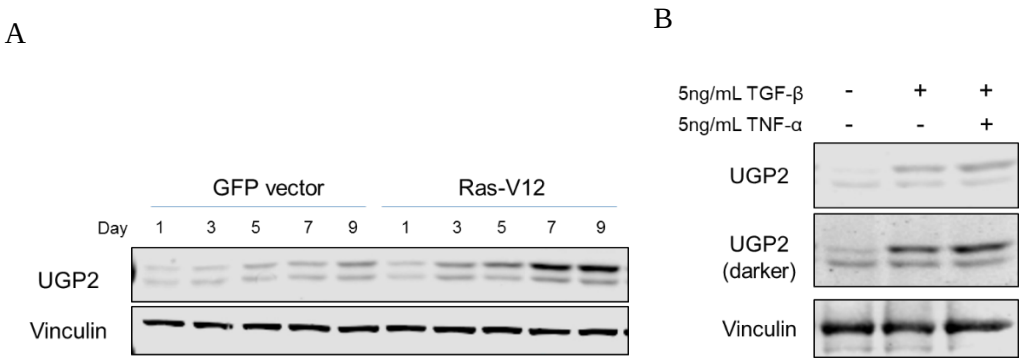


Fig. 9: Protein expression of UGP2 in MCF10A+Ras-V12 cells during the time-course DOX treatment (A) and in MCF10A cells treated with TGF-β or TGF-β and TNF-α for 10 days (B). Both experiments were repeated twice.

The immunoblotting for UGP2 after running the proteins in a SDS-PAGE gel consistently shows the presence of two specific bands. In order to assess if the bands represent phosphorylated forms of UGP2, we treated the lysates with lambda phosphatase and we concluded that the two bands are not caused by the addition of phosphate groups to the UGP2 protein (Fig. 10). Later, we determined that the two bands are actually caused by the two main isoforms of the human UGP2 (Uniprot Q16851; UniProt: the universal protein knowledgebase 2017), after selectively overexpressing the cDNA of one or the other in MCF10A cells (Fig. 11). These isoforms differ in the first 11 amino acids of the protein (presence or absence), with isoform 1 being the longer isoform and isoform 2 shorter at the N-terminus (Fig. 12). Interestingly, in the EMT models tested, ERK2-, Ras-V12-, TGF-β and TGF-β plus TNF-α, only the longer UGP2 isoform (isoform 1) was increased in the cells undergoing EMT (Figs. 8 and 9).

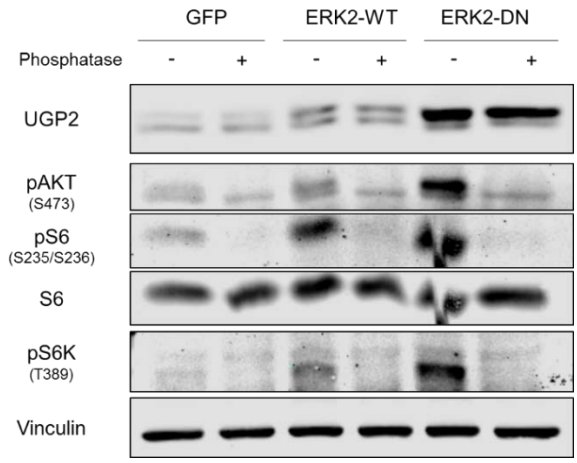


Fig. 10: UGP2 expression after treatment with lambda phosphatase in ERK2 WT and DN mutant cells compared to the vector control after 7 days of DOX treatment. The experiment was repeated twice.

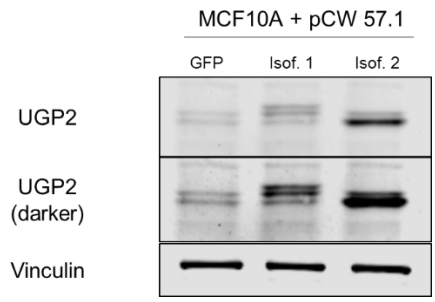


Fig. 11: Overexpression of the two UGP2 isoforms in MCF10A cells.

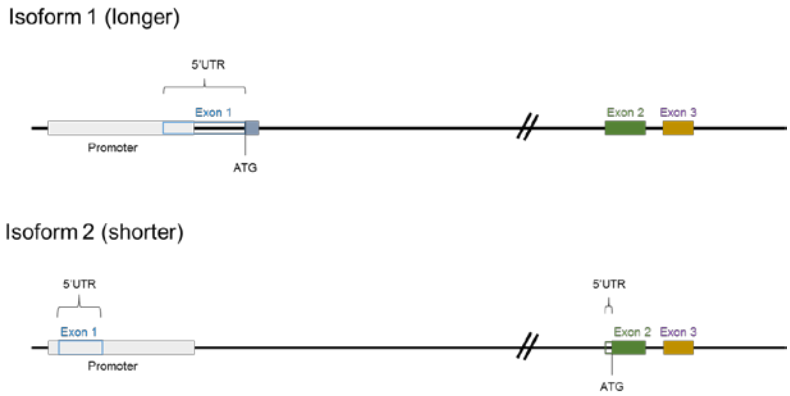


Fig. 12: Schematics for the transcripts of the isoform 1 (NM_006759.3) and isoform 2 (NM_001001521.1) of the human UGP2. The grey boxes represent the promoter and the color boxes represent the exons. The empty boxes represent non-translated exons. The 5' untranslated region (UTR) locations and the ATG site are also indicated.

UGP2 is necessary for ERK2-driven EMT

In order to assess the dependence of the ERK2-driven EMT on the UDP-glucose pathway, more specifically on the expression of UGP2, and to discriminate the events that are essential for the phenotypic transition from those that are just bystander effects, we established a model to knock-down UGP2 protein. We choose to use shRNA in order to have a stable silencing. Taking in consideration the fact that MCF10A are not easily transfectable, we used a lentivirus system to deliver the plasmids. After testing multiple constructs in different systems, we were able to knock-down UGP2 using an DOX-inducible system based on the shRNAmir technology (Dickins et al. 2005; Stegmeier et al. 2005; Zeng, Cai, and Cullen 2005; Zeng, Wagner, and Cullen 2002) with different efficiencies.

The UGP2 knock-down in cells overexpressing ERK2-DN resulted in lower levels of mesenchymal markers such as fibronectin and Slug and higher expression of E-cadherin,

compared to ERK2-DN overexpressing cells where UGP2 was not silenced (ERK2-DN + shRen) (Fig. 13). Additionally, after UGP2 silencing, ERK2-DN cells display reduced fibroblast-like morphology, more closely resembling the control epithelial cells (Fig. 14). Importantly, the increased migration and invasion capacities of ERK2-DN cells were reduced upon UGP2 knock-down (Fig. 15 and 16). Collectively, these data suggest that UGP2 plays an important role in EMT upstream of the EMT markers fibronectin and E-cadherin, as well as the EMT transcription factor Slug, consistent with its important role in EMT.

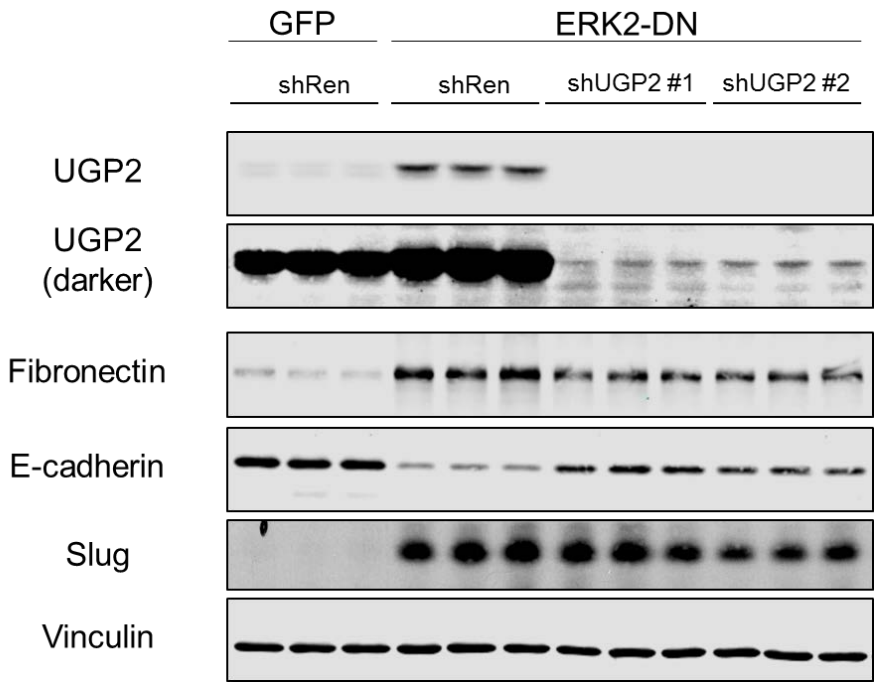


Fig. 13: Protein expression of UGP2 and select EMT markers in ERK2 DN cells infected with shRNAmir targeting UGP2 or Renilla (control), compared to GFP control, after 5 days of DOX treatment. The lanes on each condition correspond to 3 biological replicates.

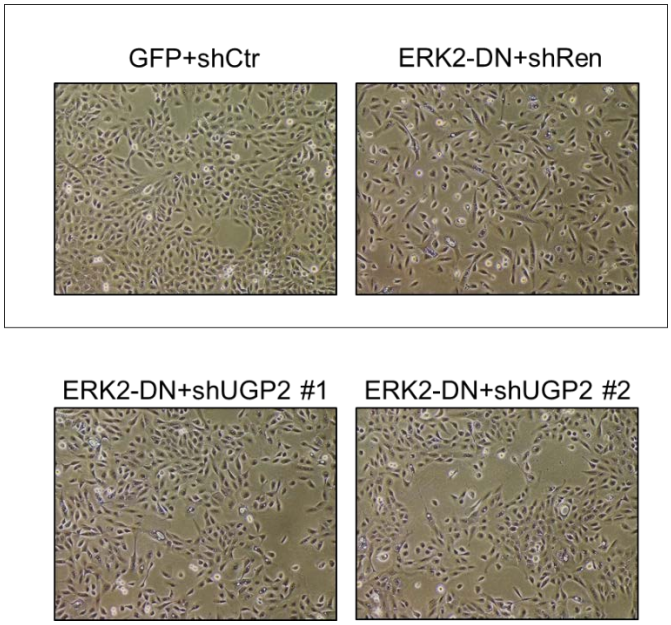


Fig. 14: Morphology of ERK2 DN cells infected with shRNAmir targeting UGP2 or Renilla (control), compared to GFP control, after 5 days of DOX treatment. The bright field images were taken with a 10x objective. The experiment was repeated 3 times.

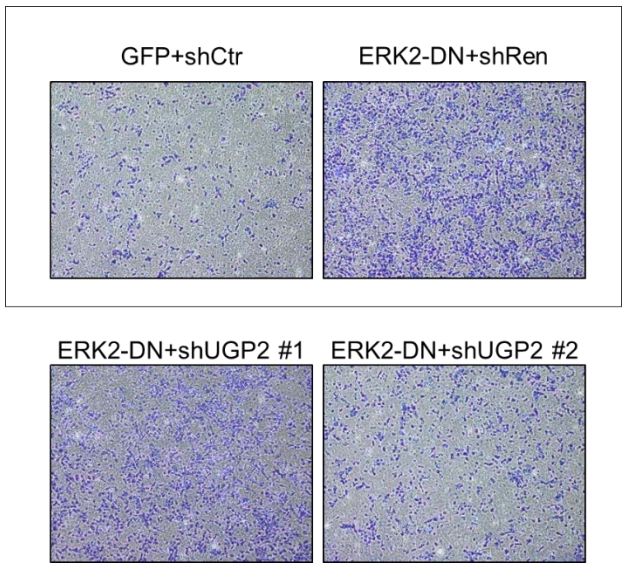


Fig. 15: Migration of ERK2 DN cells infected with shRNAmir targeting UGP2 or Renilla (control), compared to GFP control, after 5 days of DOX treatment. The experiment was repeated 3 times.

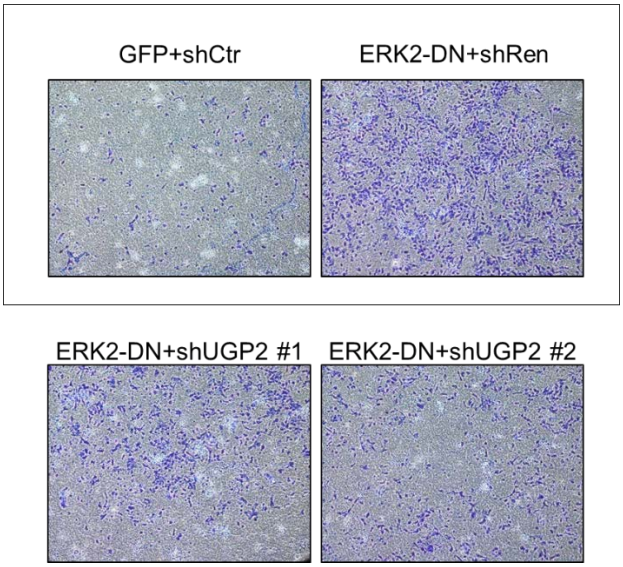


Fig. 16: Invasion in Matrigel® of ERK2 DN cells infected with shRNAmir targeting UGP2 or Renilla (control), compared to GFP control, after 5 days of DOX treatment. The experiment was repeated 3 times.

Taking in consideration the results from the knock-down of UGP2, we wanted to understand if the effects of the silencing constructs are specific by performing the "add-back" experiment. After knocking-down the endogenous UGP2 using shRNA, we overexpressed it using a plasmid with its cDNA, generated through gateway cloning. This experiment would allow us to assess if the blockage of EMT after infection with shUGP2 could be "rescued" after the enzyme re-expression, and thus be specifically due to UGP2 expression. Unfortunately, we were not able to overexpress the enzyme at the desired levels due to technical problems.

UDP-glucuronate is increased and UGDH is upregulated during EMT

UDP-glucose is a key intermediate in carbohydrate metabolism and it can have several fates inside cells (Führing et al. 2015) (Fig. 17). For example, UDP-glucose can serve as a precursor for glycogen. Thus, we analyzed the glycogen content of control and ERK2-DN cells throughout the time-course DOX treatment, using Periodic Acid–Schiff (PAS) staining, which detects polysaccharides (such as glycogen) and glycoconjugates. Although ERK2-DN cells appear to have slightly higher glycogen content on day 5 of DOX treatment, in the remaining timepoints this increase is not as evident (Fig. 18). Since the results from this assay were not conclusive, we explored other pathways associated with UDP-glucose.

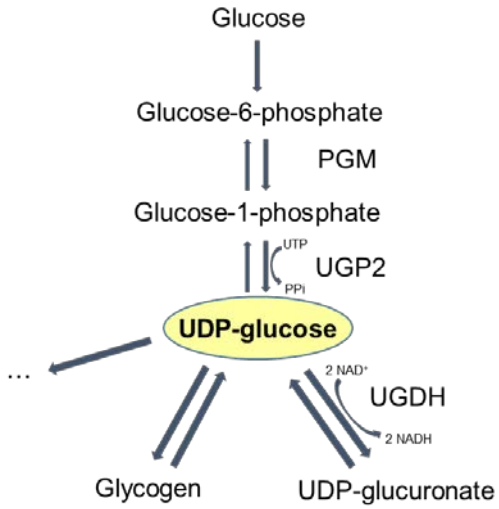


Fig. 17: Schematics with the 2 more relevant products of UDP-glucose.

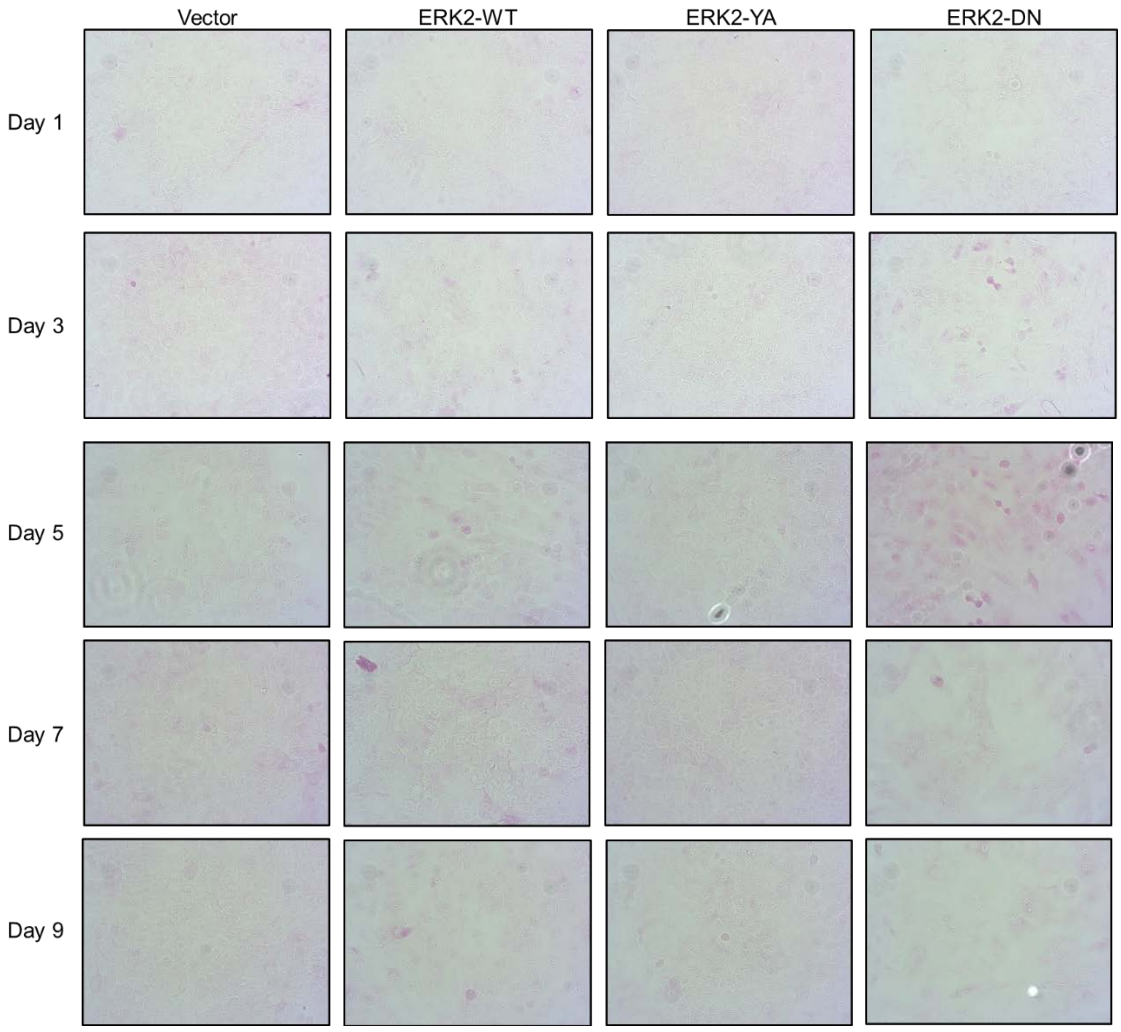


Fig. 18: Glycogen content assessment using PAS staining in ERK2-WT, -YA and -DN cells compared to the vector control, during the time-course DOX treatment.

UDP-glucose can also be converted into UDP-glucuronate through UDP-glucose 6-dehydrogenase (UGDH). From the metabolomics data, ERK2-DN cells on day 7 of DOX treatment exhibited higher levels of UDP-glucuronate compared to the controls (Fig. 19).

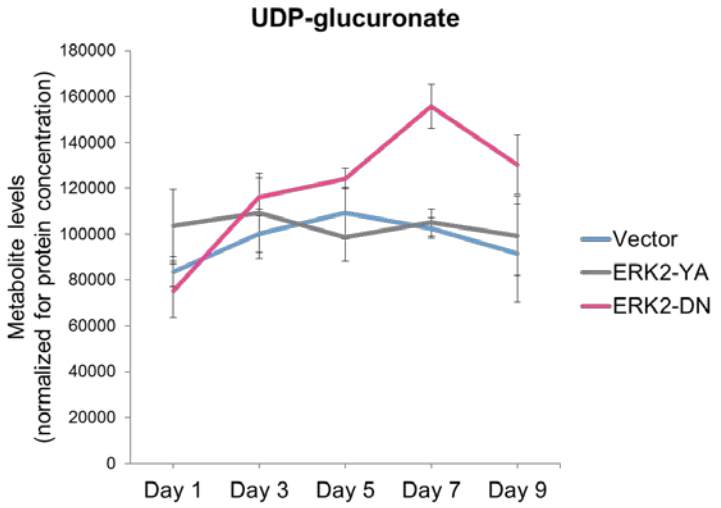


Fig. 19: UDP-glucuronate levels in ERK2 YA and DN cells compared to the vector control during the time-course DOX treatment. Quantification of integrated peak areas TIC values. The experiment was repeated 3 times. Data are represented as mean ± SD.

The analysis of the mRNA and protein levels of UGDH shows that this enzyme was also upregulated in cells undergoing EMT (Fig. 20). This may be of particular relevance to mesenchymal cells because UDP-glucunorate is a precursor for proteoglycans and glycosaminoglycans, such as hyaluronan, which compose the extracellular matrix and are important for migration and invasion of cancer cells (Nikitovic et al. 2014).

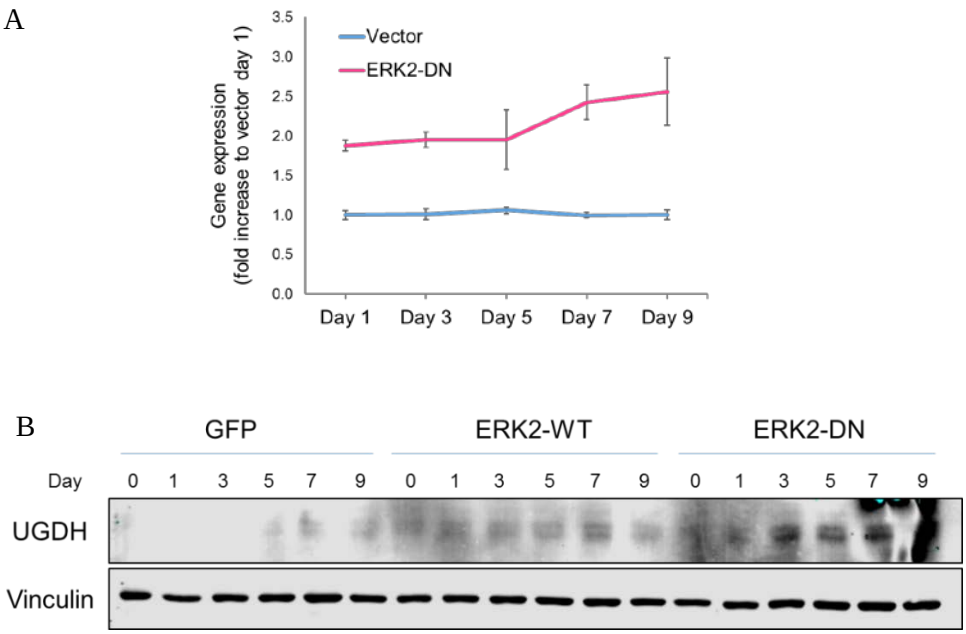


Fig. 20: Expression of UGDH at the mRNA (A) and protein (B) level in ERK2 WT and DN cells compared to the vector control during the time-course DOX treatment. Both experiments were repeated at least 3 times. mRNA data are represented as mean ± SD.

Hyaluronan may be mediating the effects of UDP-glucose and hexosamine biosynthetic pathway in EMT

From the previous results, we concluded that mesenchymal cells that overexpress the DN mutation in ERK2 have increased levels of UDP-glucose and show upregulation of the enzyme that catalyzes its production, UGP2. Moreover, UDP-glucuronate, a metabolite produced from UDP-glucose and the enzyme responsible for its production, UGDH, are also increased. As such, we explored how the UDP-glucose pathway may impact the mesenchymal phenotype, especially the enhanced migration and invasion. One possible link is through the generation of hyaluronan (HA), a polysaccharide synthetized by HA synthases (HAS) using cytosolic UDP-glucuronate and UDP-N-acetylglucosamine (UDP-GlcNAc) as precursors (Weissmann et al. 1954). Indeed, MCF10A cells that are undergoing EMT also showed increased levels of UDP-GlcNAc, the end-product of the hexosamine biosynthetic pathway (Fig. 21). Because this metabolite can also be used in the glycosylation of proteins (both N- and O-glycosylation) and in the formation of glycosaminoglycans (Pinho and Reis 2015), we analyzed the glycosylation pattern of the global cellular proteome using an O-GlcNAc antibody for O-glycosylation and probing total cellular extracts with lectins, which are carbohydrate-binding proteins that bind to specific forms of glycans (Cummings and Etzler 2009). We did not find global differences in the pattern of

glycosylated proteins when comparing ERK2-DN with control cells, although minor changes may exist for PHA-E and O-glycosylation (Fig. 22).

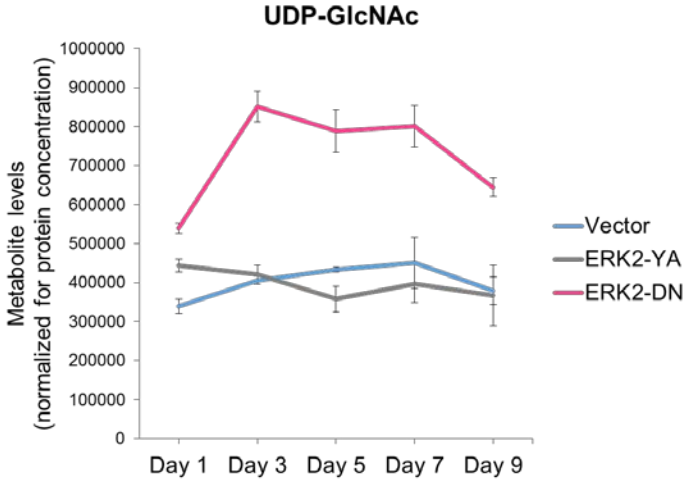


Fig. 21: UDP-GlcNAc levels in ERK2 YA and DN mutants compared to the vector control during the time-course DOX treatment. Quantification of integrated peak areas TIC values. The experiment was repeated 3 times. Data are represented as mean ± SD.

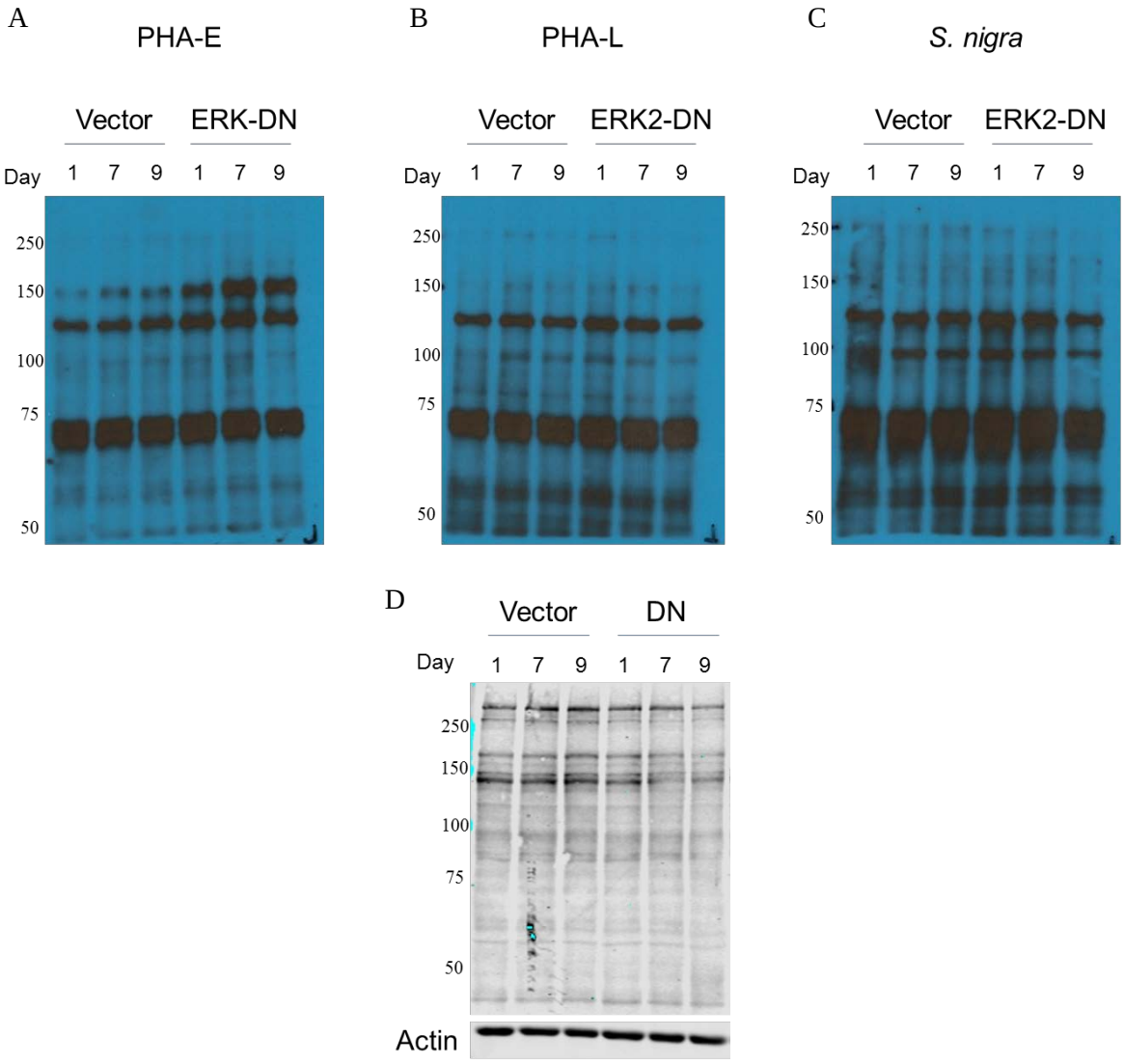


Fig 22: Expression of proteins with the N-glycan and O-glycans epitopes in ERK2-DN cells compared to the vector control after 1, 7 and 9 days of DOX treatment, using lectins and an O-GlcNAc antibody. The lectins *Phaseolus vulgaris* erythroagglutinin (PHA-E) detects bisecting GlcNAc N-glycan structures (A), *Phaseolus vulgaris* leucoagglutinin (PHA-L) detects β1,6 GlcNAc-branched N-glycan structures (B) and *Sambucus nigra* lectin recognizes α2,6; α2,3 linked sialic acid (C). O-Glycans were recognized using an O-GlcNAc antibody (D).

In accordance with the increased UDP-GlcNAc levels, we identified UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) - the enzyme that catalyzes the production of UDP-GlcNAc – as being upregulated in ERK2-DN cells at the mRNA and protein level after DOX treatment (Fig. 23). In ERK2-WT cells after DOX treatment, UAP1 protein is also increased (Fig. 23B).

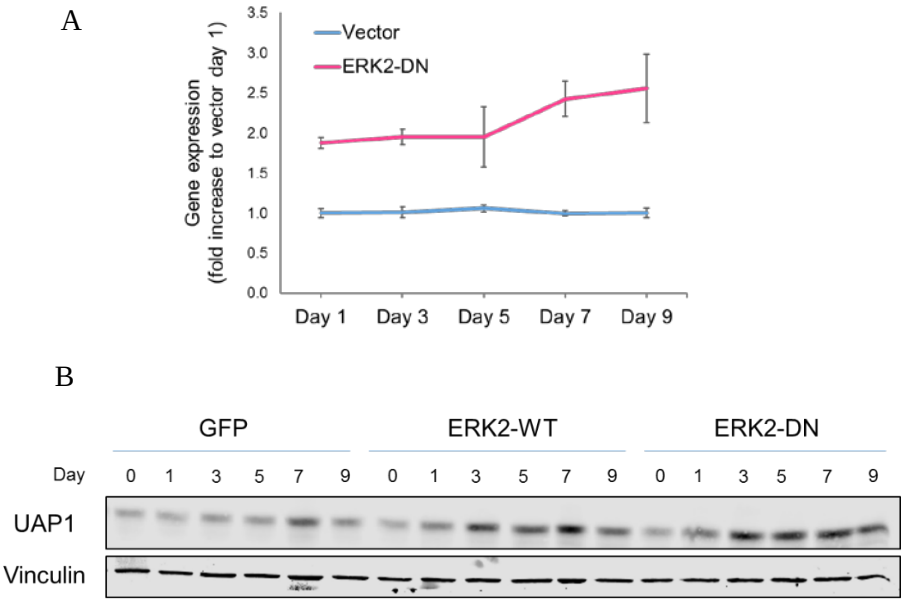


Fig. 23: Expression of UAP1 at the mRNA (A) and protein (B) level in ERK2 -WT and -DN cells compared to the vector control during the time-course DOX treatment. Both experiments were repeated at least 3 times. mRNA data are represented as mean ± SD.

We decided to focus our attention in HA, which can be produced by 3 HA synthases localized at the plasma membrane. One of these - hyaluronan synthase 2 (HAS2) – was found to have increased mRNA levels in ERK2-DN cells (Fig. 24). Unfortunately, the protein levels were not measured because after troubleshooting the conditions, neither of the several antibodies tested did not work for immunoblotting.

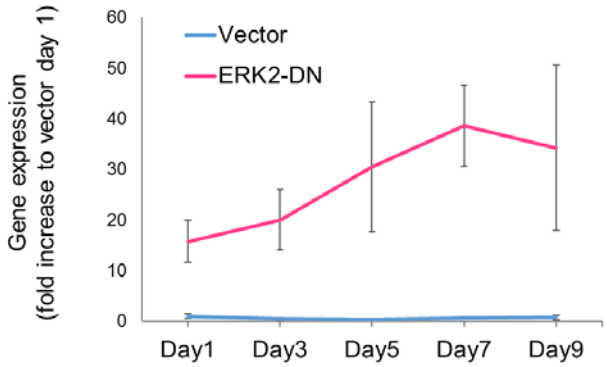


Fig. 24: mRNA expression of HAS2 in ERK2-DN mutant cells compared to the vector control during the time-course DOX treatment. The experiment was repeated twice. Data are represented as mean ± SD.

Since our findings suggest an enrichment of the metabolic intermediates that lead to HA production, we next determined if the knock-down of HAS2 impacts the mesenchymal phenotype. We were able to identify 3 shRNAs that efficiently silence at least 50% of HAS2 in ERK2-DN overexpressing cells (Fig. 25). We then looked for the impact of HAS2 silencing on HA levels, using HA binding protein (HABP), due to the lack of consistent Western blotting results. Our result show that HAS2 silencing in MCF10A cells treated with TGF-β (4 days of treatment) results in decreased levels of HA (Fig. 26).

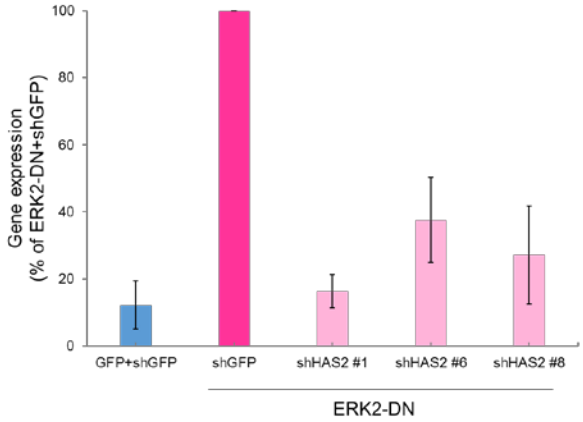


Fig. 25: mRNA expression of HAS2 in ERK2-DN cells infected with shRNAs targeting HAS2 or control, compared to GFP control, after 5 days of DOX treatment. The experiment was repeated 3 times. Data are represented as mean ± SD.

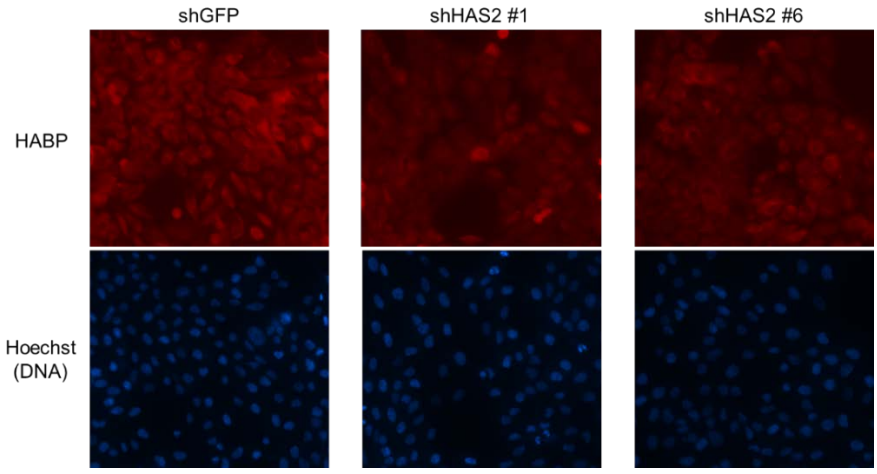


Fig. 26: HABP staining (red) on MCF10A cells infected with shRNAs targeting HAS2 or control treated with TGF-β for 4 days, compared to control cells. The nucleus was counterstained with Hoechst (blue) for better perception of the cell number and localization. The immunofluorescence images were taken with a 20x objective and were digitally zoomed 3 times.

When inducing ERK2-DN overexpression for 5 days in cells silenced for shHAS2, we observed that the levels of E-cadherin are not as decreased nor the levels of fibronectin as increased as in ERK2-DN cells that express HAS2 (Fig. 27). Besides, with HAS2 knock-down, ERK2-DN cells look less like fibroblasts and migrate less that the cells infected with the shRNA control (Figs. 28 and 29), confirming that HAS2 expression has a role in the maintenance of EMT.

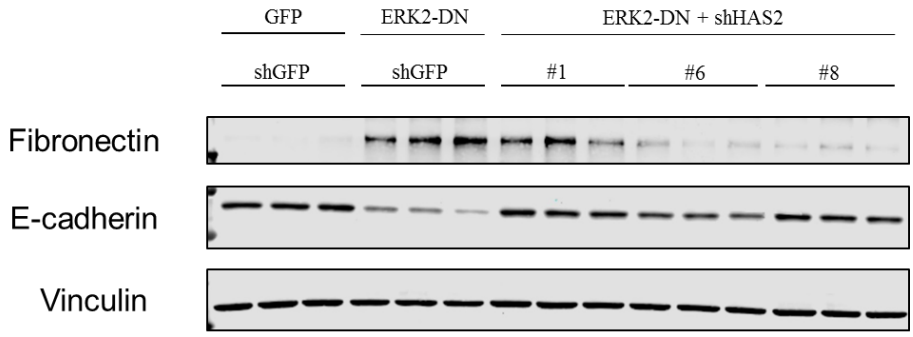


Fig. 27: Protein expression of selected EMT markers in ERK2 DN cells infected with shRNAs targeting HAS2 or GFP, compared to GFP control, after 5 days of DOX treatment. The lanes on each condition correspond to 3 biological replicates.

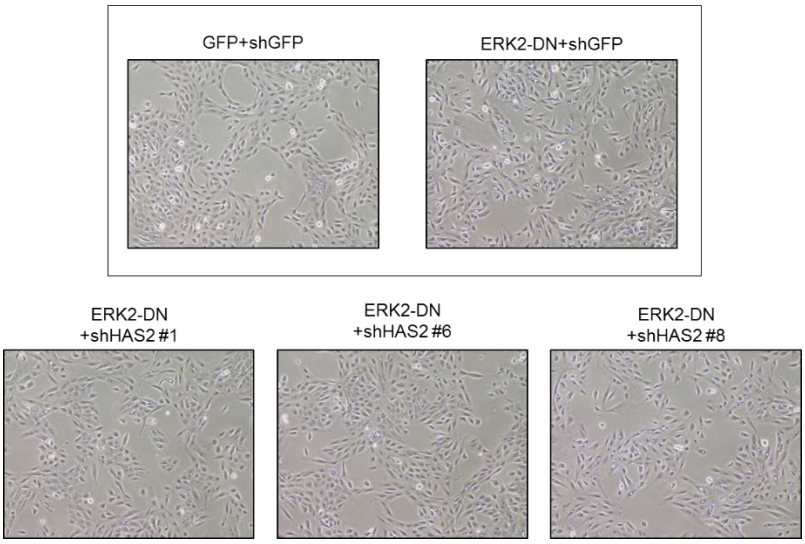


Fig. 28: Morphology of ERK2 DN cells infected with shRNAs targeting HAS2 or GFP, compared to GFP control, after 5 days of DOX treatment. The bright field images were taken with a 10x objective. The experiment was repeated 3 times.

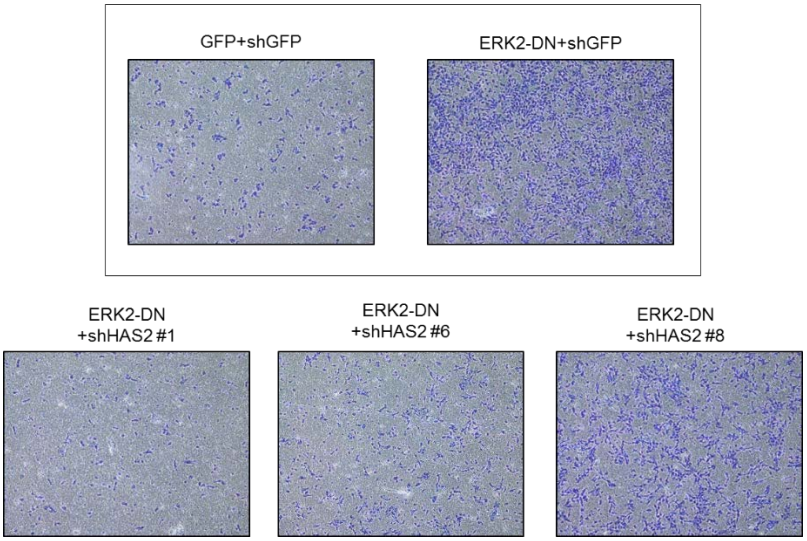


Fig. 29: Migration of ERK2 DN cells infected with shRNAs targeting HAS2 or GFP, compared to GFP control, after 5 days of DOX treatment. The experiment was repeated 3 times.

Promoter activity does not explain the upregulation of UGP2

UGP2 is overexpressed in cells undergoing EMT after expression of a mutated form of ERK2. However, it is unclear how ERK2 signaling leads to altered expression of the metabolic enzyme. As shown earlier by qPCR, the mRNA levels of UGP2 are increased in ERK2-DN cells (Fig. 8), so, since the most-well studied ERK substrates are TFs, a possible explanation for this a higher activity of the UGP2 promoter in these cells. So, we performed a luciferase promoter assay, by transfecting ERK2 -WT and -DN cells with a plasmid that contains the gene encoding luciferase under the UGP2 promoter: in this assay, the more active the promoter is, the more luminescent the cells become. Surprisingly, the promoter for UGP2 used in this assay seems to be less active in DN cells than in control and ERK2-WT cells (Fig. 30), suggesting that the increased mRNA and protein levels of UGP2 in ERK2 WT and DN cells are not due to increased promoter activity.

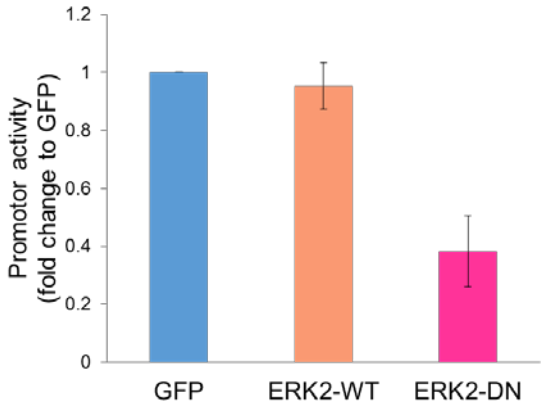


Fig 30: Promoter activity analysis of ERK2 WT and DN cells transfected with luciferase constructs under the promoter of UGP2, compared to control GFP cells, after 5 days of DOX treatment. Promoter activity measured by *Renilla* luciferase was normalized by *Cypridina* luciferase and to MCF10A+GFP cells. The experiment was repeated 3 times. Data are represented as mean ± SD.

Treatment with a MAPK inhibitor does not affect significantly the UGP2 protein levels

Since UGP2 is induced after an increase in ERK2 signaling through the DEF-motif, we tested if a MEK inhibitor, the upstream kinase of ERK, could lower UGP2 levels. However, a 4-hour treatment with AZD6244 (Yeh et al. 2007), which has an impact on ERK1/2 phosphorylation, did not significantly alter the expression of UGP2 in ERK2-WT, -DN nor in GFP control cells (Fig. 31). This could be related to the duration of the treatment and so a longer treatment should be tested.

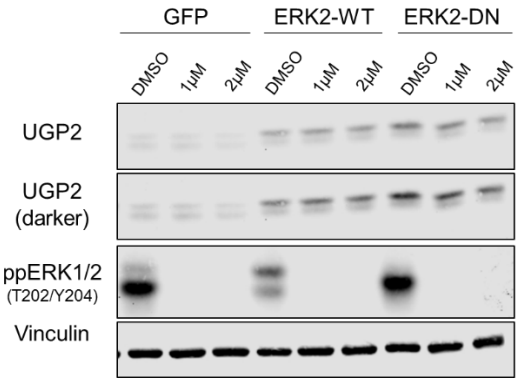


Fig. 31: Protein expression of UGP2 in ERK2 WT and DN cells after 7 days, compared to control cells of DOX treatment, treated for 4h with 1µM or 2 µM of AZD6244, a MEK1 inhibitor (or DMSO control). ppERK1/2 expression was used as a control.

Association between UGP2 mRNA and relapse free survival of breast cancer patients

To understand if UGP2 expression is associated with prognosis in breast cancer patients we used the Kaplan Meier plotter, a software that combines gene expression data and patients prognosis information (Lánczky et al. 2016). We saw that higher mRNA expression of UGP2 is significantly associated with poor relapse free survival in a breast cancer patient cohort (Fig. 32).

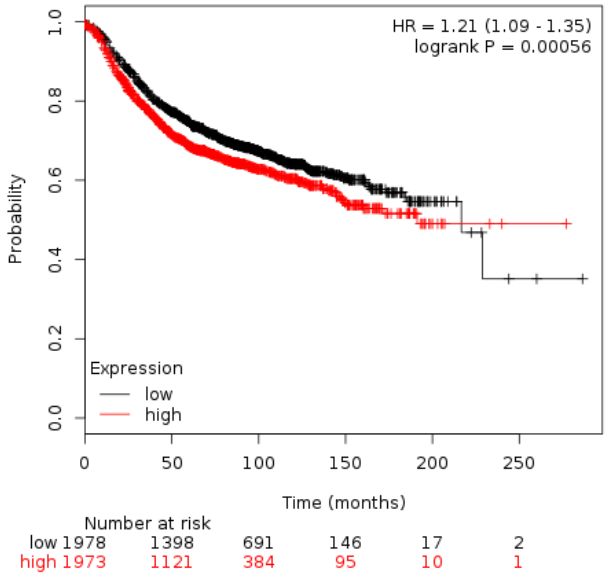


Fig. 32: Kaplan-Meier survival plot from breast cancer patients with high and low levels of UGP2 expression. Gene expression data and relapse information are downloaded from GEO (Affymetrix microarrays only), EGA and TCGA. The database is handled by a PostgreSQL server, which integrates gene expression and clinical data simultaneously. To analyze the prognostic value of a particular gene, the patient samples are split into two groups according to various quantile expressions of the proposed biomarker. The two patient cohorts are compared by a Kaplan-Meier survival plot, and the hazard ratio with 95% confidence intervals and logrank P value are calculated. Each database is updated biannually. Data from the open source Kaplan Meier plotter for Breast Cancer (Lánczky et al. 2016).

Discussion

In this work, we identified a link between UDP-glucose metabolism and the acquisition of mesenchymal properties driven by ERK2, in the context of cancer-associated EMT. Specifically, UGP2, the enzyme that produces UDP-glucose, is essential for the acquisition of a mesenchymal phenotype in cells overexpressing ERK2-DN. This may be mediated by the production of HA, through HAS2.

With the goal of unravelling the metabolic requirements of EMT, we took advantage of a previously characterized model where EMT is induced by the signaling downstream of the DEF motif of ERK2 in mammary epithelial cells (Shin, Dimitri et al. 2010). We used a DOX-inducible approach for a controlled induction of ERK2 expression, enabling us to map the resulting molecular alterations over time. This model allowed us to uncover interesting alterations in the metabolic landscape during EMT, in agreement with recent studies also reporting links between metabolism and EMT, using other model systems. A metabolic reprogramming was shown when EMT is induced by TGF-β signaling (Soukupova et al. 2017) or Snail overexpression (Bhowmik et al. 2015; N. H. Kim et al. 2017), and this last one also leads to differential expression of several metabolic enzymes (Bhowmik et al. 2015; De Craene et al. 2005). Moreover, a few direct links between metabolic enzymes and EMT effectors have already been found. For example, Snail binds directly and regulates the expression of fructose-1,6-bisphosphatase and phosphofructokinase (Dong et al. 2013; Kim et al. 2017).

The metabolic reprogramming of cells undergoing ERK2-driven EMT is characterized by alterations in the UDP-glucose pathway. Specifically, the levels of UDP-glucose become heightened, together with an upregulation of UGP2, the only human enzyme that catalyzes UDP-glucose production. Importantly, we found that UGP2 is required for the maintenance of the mesenchymal phenotype. To the best of our knowledge, no studies have addressed the role of UDP-glucose and UGP2 in EMT and previous work has been limited to descriptions of their increased levels in EMT models (Lucena et al. 2016; Mulvihill et al. 2014). In a study by Mulvihill *et al.*, a metabolomics approach in several *in vitro* breast models of cellular transformation and malignant progression, driven by the HRAS and Hippo pathways, identified UDP-glucose as one of the metabolites predominantly increased in cells undergoing EMT (Mulvihill et al. 2014). UGP2 was also found to be upregulated in a proteomics analysis of the same study (Mulvihill et al. 2014) as well as in a TGF-β-driven EMT model of lung cancer cells (Lucena et al. 2016). UDP-glucose is also increased in a Twist-induced EMT model (Bhowmik et al. 2015). These reports suggest a potentially important role for UGP2 for the acquisition of migration properties during the first step of metastasis. In fact, UGP2 was considered a biomarker in gallbladder cancer patients, since its expression was associated with invasion, metastasis and poor patient prognosis.

Besides, UGP2 was frequently associated with positive expression of vimentin, MMP-9, MMP-2 and β-catenin (Wang et al. 2016). However, in hepatocellular carcinoma patients, UGP2 was included in a panel of potential markers that distinguish “metastatic relapse” and “non-relapse” patients, being downregulated in the primary tumors of the “metastatic relapse” patients. Interestingly, UGP2 is highly expressed in non-tumor liver tissue (Tan et al. 2014), which might explain such apparently contradictory findings.

We noticed that UGP2 has two main isoforms in MCF10A cells, being the longer isoform the one that is upregulated. Interestingly, alternative transcription start site usage of UGP2 has been identified in colorectal cancer patient samples (both adenoma and carcinoma) relative to normal mucosa (Thorsen et al. 2011). Although the authors do not provide further information, it is possible that this reflects the two isoforms that we describe in the model presented on fig. 12 and if so, it may indicate that their expression is regulated in tumors.

To understand how UGP2 expression is regulated in ERK2-DN cells, we performed a promoter assay that, contrarily to our expectations, showed a less active UGP2 promoter in cells undergoing EMT than in controls. To note that the promoter sequence used in the assay is expected to be regulating the transcription of the longer UGP2 isoform, the one that is upregulated during EMT. The apparent lack of consistency between upregulated UGP2 mRNA levels and lower UGP2 promoter activity can be attributed to the promoter sequence used in this assay that, although having an approximate length of 1kb, may not include all the regulatory sequences for UGP2 transcription. However, if our results are confirmed, an increased stability of UGP2 mRNA in cells undergoing EMT would also explain the higher mRNA levels. This can be tested by treating cells with an inhibitor of transcription (actinomycin D) and collecting mRNA in several time-points to analyze its stability over time. Indeed, some ERK2 substrates may regulate RNA stability (Cargnello and Roux 2011). Additionally, we used a MEK inhibitor in order to evaluate the effect of blocking ERK pathway on UGP2 levels. This preliminary experiment showed that, after 4h of treatment, although the ERK pathway is efficiently inhibited, there were no striking effects on UGP2 protein expression. However, it is important to point that we do not know if, in the conditions used, UGP2 mRNA levels are affected by MEK inhibition and how stable UGP2 mRNA is in these cells. Besides, these results should be interpreted with caution since we need to understand the proteins that are mediating the upregulation of UGP2 by ERK2-DN signaling and their turnover rates. So, a time-course analysis of UGP2 mRNA and protein levels including longer treatments with the MEK inhibitor should be performed. Alternatively, we could also perform an experiment where DOX is washed out from the cells and analyze UGP2 turnover.

Our results further suggest that the UDP-glucose pathway provides substrates that contribute for the mesenchymal phenotype. UDP-glucose is the form by which glucose is incorporated into the

glycogen molecule. A higher glycogen content has been associated with brain metastasis of human breast cancer patients, when compared to the primary tumor, although the analysis was only performed in 5 brain metastasis samples (Chen et al. 2015). In our study, however, glycogen content of the mesenchymal cells was not significantly altered when compared with epithelial cells. An alternative link between UDP-glucose and EMT highlighted in our study is the convergence of UDP-glucose metabolism and hexosamine biosynthetic pathway into HA. HA is a polymer of glucunorate, a product of UDP-glucose, and GlcNAc; the precursors metabolites - UDP-glucuronate and UDP-GlcNAc – were also found increased in cells acquiring mesenchymal properties. Accordingly, UGDH and UAP1, the enzymes that produce both precursors of HA molecule, are upregulated in cells undergoing EMT, warranting further studies aiming at understanding if these enzymes are also required for ERK2-driven EMT. Although HA levels in ERK2-DN overexpressing cells could not be measured due to methodological limitations (sensitivity of the technique), UDP-sugar availability in the cytosol can impact HA synthesis (Jokela et al. 2008; Vigetti et al. 2014). It has also been already shown that overexpression of UGP2 and UGDH leads to an increase of HA accumulation (Magee, Nurminskaya, and Linsenmayer 2001; Vigetti et al. 2006). Thus, we anticipate that silencing of the enzymes involved in UDP-sugar production will lead to decreased levels of HA. HA is associated with cancer cell microenvironment, playing an important role in tumor progression (Toole, Biswas, and Gross 1979). A recent publication using breast cancer patient biopsies showed that UDP-glucose, UDP-glucuronate and UDP-GlcNAc are increased, and UDP-GlcNAc and UDP-glucuronate correlate with the increased hyaluronan levels (Oikari et al. 2018). Several studies have associated HA with EMT. For example, when cells are treated with HGF or overexpress β-catenin, the resulting increased invasion and anchorage-independent growth are reversed by perturbation of endogenous hyaluronan polymer interactions, by treatment with hyaluronan oligomers (Zoltan-Jones et al. 2003). In TGF-β-induced EMT, HA production is increased, in a process dependent on the upregulation of HAS2 and on the activation of the p38 MAPK. HAS2 is the most abundant HA synthase in adult cells, produces high molecular weight HA (Itano et al. 1999) and has an active role on EMT during development (Camenisch et al. 2000). Additionally, HAS2 is in part required for the increased cell migration triggered by TGF-β in mouse mammary epithelial cells, although, intriguingly, this seems to be independent of CD44 – a cell membrane receptor that can bind HA – or of HAS2 enzymatic activity to synthetize HA (Porsch et al. 2013). Moreover, increasing the hyaluronan content by overexpressing HAS2 in breast and kidney epithelial cells, is sufficient to induce EMT in normal epithelial cells (Zoltan-Jones et al. 2003).

HAS2 is associated with several poor prognostic features (such as higher grade, lymphovascular invasion, basal-like breast cancer subtype, high cell proliferation and basal marker expression) in androgen receptor negative breast cancer. The adverse prognostic effect of the androgen receptor

negative and HAS2 positive expression was independent of the expression of CD44 (Zhang et al. 2016).

Future studies aiming at clarifying the role of HA in ERK2-DN cells are needed. For example, whether EMT blockage caused by silencing of UGP2 or HAS2 occurs in a HA-dependent manner can be assessed by rescuing cells with HA or conditioned media from mesenchymal cells (Goncharova et al. 2012). Besides, HA can bind several receptors at the cell membrane, including the CD44 receptor, so we hope that further tests will indicate if CD44 is mediating the effects of HA on EMT. The CD44 surface glycoprotein has multiple isoforms, generated by alternative splicing, being the short CD44 isoforms associated with a mesenchymal phenotype upon EMT induction (Brown et al. 2011). In ovarian cancers, binding of HA to CD44 leads to the activation of ERK signaling, in a process where ERK2 is phosphorylated, resulting in Elk-1 phosphorylation and activation (Bourguignon et al. 2005). Interestingly, HAS2, as well as the other HAS isoforms, have been shown to be phosphorylated and activated by ERK, resulting in increased HA production in human ovarian tumor cells (Bourguignon, Gilad, and Peyrollier 2007). The receptor for hyaluronic acid-mediated motility (RHAMM) also contributes to activation of ERK and for CD44-ERK mediated signaling required for wound repair (Tolg et al. 2006; Zhang et al. 1998).

On the other hand, UDP-glucose itself (and other UDP sugars such as UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine) can be secreted, bind and activate the $P2Y_{14}$ receptor, a seven-transmembrane-spanning G protein-coupled receptor, leading to a signaling cascade that results in the activation of MAPK signaling pathway through ERK1/2 phosphorylation (Chambers et al. 2000; Fricks et al. 2009). Moreover, $P2Y_{14}$ receptor activation by UDP-glucose also enhances transcription of HAS2 and HA production (Jokela et al. 2014). Finally, extracellular UTP also increases pericellular HA and induces HAS2 expression, in a process mediated in part by ERK (Jokela et al. 2017). As such, the role of UDP-glucose and HA in EMT may be explained by their signaling properties, which occur *via* the interaction with cell surface receptors, ultimately leading to ERK2 activation, in positive feedback loop that sustains ERK activation, being crucial in maintaining the mesenchymal commitment of the cells.

We are aware that a downside of our metabolomics approach is that it only gives us the steady state picture of the metabolites' levels at the moment of collection. For a better notion of the glucose fate, we would have to perform a flux experiment by growing the cells in ^{13}C-labeled glucose and tracing the labeled carbons in the product metabolites. The metabolomics approach will also allow us to analyze the levels of the UDP-glucose pathway metabolites after UGP2 silencing.

Our results stimulate interesting hypotheses: is ERK2 regulating the fine tuning of UDP-glucose fate? Is ERK2 coordinating the expression of the different enzymes of UDP-sugar metabolism,

that lead to the production of HA? These thoughts warrant further research in order to fully understand the relationship between EMT, ERK2 and metabolism

Taken together, our results provide further evidence that metabolism is important for cancer progression, specifically for the acquisition of migration properties that facilitate the initiation of metastasis formation. Our results also indicate that UDP-glucose pathway is one of the targets of ERK2 signaling.

Acknowledgements

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Supplementary data

Table 1: List of antibodies and binding proteins

Protein	Source	Identifier	Clonality	Species	Dilution	Protein size (kDa)
HA	SCBT	sc-7392	Monoclonal	mouse	1:5000	
E-cadherin	BD Transduction Laboratories	610181	Monoclonal	mouse	1:3000	120
Fibronectin	BD Transduction Laboratories	610077	Monoclonal	mouse	1:1000	240
Slug						
UGP2	Abcam	ab157473	Monoclonal	rabbit	1:5000	50
UGDH	SCBT	sc-137005	Monoclonal	mouse	1:500	57
UAP1	Sigma	HPA014659	Polyclonal	rabbit		60
ppERK1/2 (T202/Y204)	CST	4370	Monoclonal	rabbit	1:1000	44 and 42
pAKT (S473)	CST	4060	Monoclonal	rabbit	1:1000	60
pS6 (S235/S236)	CST	2211	Polyclonal	rabbit	1:1000	32
S6	CST	2317	Monoclonal	mouse	1:1000	32
pS6K (T389)	CST	9234	Monoclonal	rabbit	1:1000	70, 85
Glut1	Abcam	ab652	Polyclonal	rabbit	1:1000	55
O-GlcNAc	SCBT	sc-59624	Monoclonal	mouse	1:1000	N/A
Vinculin	Sigma-Aldrich	V9264	Monoclonal	mouse	1:5000	116
Actin	SCBT	sc-1615	Polyclonal	goat	1:1000	43
Alexa Fluor 488 goat anti-mouse IgG (H+L)	Thermo Fisher Scientific	A11001	Polyclonal	goat	1:1000	N/A
Streptavidin, Alexa Fluor™ 568 conjugate	Thermo Fisher Scientific	S11226	N/A	N/A	1:1000	N/A
Hyaluronic Acid Binding Protein-Biotin bovine	Sigma-Aldrich	H9910	N/A	bovine	10µg/mL	N/A

Table 2: List of lectins

Lectin	Antigen	Source	Identifier	Concentration
PHA-E	Galβ4GlcNAcβ2Manα6 (GlcNAcβ4) (GlcNAcβ4Manα3) Manβ4	Vector Laboratories	B-1125	0.5µg/mL
PHA-L	Galβ4GlcNAcβ6 (GlcNAcβ2Manα3) Manα3	Vector Laboratories	B-1115	0.5µg/mL
Sambucus nigra lectin	NeuAcα2,6Gal- or NeuAcα2,6GalNAc-	Vector Laboratories	B-1305	0.25µg/mL

Table 3: List of critical commercial assays

Critical commercial assays	Source	Identifier
RNeasy Mini Kit	QIAGEN	74106
PureLink™ RNA Mini Kit	Ambion	12183018A
iScript™ cDNA Synthesis Kit	Bio Rad	1708891
DC™ Protein Assay Kit II	Bio-Rad	5000112
Periodic Acid-Schiff (PAS) Kit	Sigma-Aldrich	395B
Gateway™ LR Clonase™ II Enzyme mix	Invitrogen	11791100
Gateway™ BP Clonase™ II Enzyme mix	Invitrogen	11789100
LightSwitch™ Dual Assay Kit	Active Motif	32035
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen	K210011
E.Z.N.A.® Cycle Pure Kit (V-spin)	Omega	D6492
VECTASTAIN® Elite® ABC-HRP Kit (Peroxidase, Standard)	Vector Laboratories	PK-6100

Table 4: List of chemicals, peptides and recombinant proteins

Chemicals, peptides and recombinant proteins	Source	Identifier
Doxycyclin	Calbiochem	324385
Recombinant human TGF-β1	PeproTech	100-21
Recombinant human TNF-α	PeproTech	300-01A
Recombinant human EGF	PeproTech	AF-100-15
Insulin (human)	Sigma-Aldrich	I9278
Cholera toxin from *Vibrio cholerae*	Sigma-Aldrich	C8052
Hydrocortisone	Sigma-Aldrich	H4001
Puromycin dihydrochloride from *Streptomyces alboniger*	Sigma-Aldrich	P7255
G418 disulfate	Caisson Labs	G030
Hoechst 33258, Pentahydrate (bis-Benzimide)	ThermoFisher Scientific	H3569
Lipofectamine® 2000 Transfection Reagent	Invitrogen	11668500
DNase I Amplification Grade	Sigma-Aldrich	AMP01
Lambda Protein Phosphatase (Lambda PP)	New England BioLabs	P0753
X-tremeGENE™ HP DNA Transfection Reagent	Roche	06366546001
Odyssey® Blocking Buffer (TBS)	LI-COR	927-50150
SYBR™ Green PCR Master Mix	Life Technologies	4334973
Platinum™ Pfx DNA Polymerase	Invitrogen	11708013
T4 DNA Ligase	New England BioLabs	M0202S

Table 5: Cell culture media and serum

Cell media and serum	Source	Identifier
Dulbecco's Modification of Eagle's Medium (DMEM) / Ham's F-12 50/50 Mix containing L-glutamine and 15 mM HEPES	Corning	10-092
DMEM, high glucose	Gibco	11965118
Horse serum	Gibco	16050122
Fetal bovine serum	Sigma-Aldrich	F6765

Table 6: List of oligonucleotides and recombinant DNA

Oligonucleotides and recombinant DNA	Source	Identifier
Lentiviral packaging and envelope plasmids	Dr. David Baltimore	N/A
pTRIPZ-ERK2-YA	This paper	N/A
pTRIPZ-ERK2-YA	This paper	N/A
pINDUCER-ERK2-WT	This paper	N/A
pINDUCER-ERK2-YA	This paper	N/A
pINDUCER-ERK2-DN	This paper	N/A
pRRL-Lenti-miRE-Ren713	Dr. Lukas Dow	N/A
pRRL-Lenti-miRE-Ren713-shUGP2 #1	This paper	N/A
pRRL-Lenti-miRE-Ren713 -shUGP2 #2	This paper	N/A
pRRL-Lenti-miRE-Ren713 -shRenilla 713	Dr. Lukas Dow	N/A
pLKO.1-puro-shHAS2 #1	Broad Institute	TRCN0000045393
pLKO.1-puro-shHAS2 #6	Broad Institute	TRCN0000045396
pLKO.1-puro-shHAS2 #8	Broad Institute	TRCN0000418983
pLKO.1-puro-shGFP	Broad Institute	TRCN0000072181
pOTB7 UGP2 isoform 2	PlamID - Harvard plasmid	HsCD00325250
pENTR223 UGP2 isoform 1	PlamID - Harvard plasmid	HsCD00376643
pENTR-GFP	Addgene	15301
pCW57.1 destination vector	Addgene	41393
pCW57.1-UGP2 isoform 1	This paper	N/A
pCW57.1-UGP2 isoform 2	This paper	N/A
pCW57.1-GFP	This paper	N/A
Cypridina TK control promoter construct	SwitchGear Genomics	SN0322S
LightSwitch™ UGP2 promoter construct	SwitchGear Genomics	S708263

Table 7: List of other reagents

Other reagents	Source	Identifier
Transparent PEM Membrane 24-well 8.0 µm pore size	VWR	62406-198
Cultrex® 5X BME Solution	Trevigen	3455-096-02
Matrigel® Invasion Chamber 24-well Plate 8.0 Micron	Corning	354483
Nitrocellulose membranes	GE Healthcare	10600002

Table 8: List of qPCR primers

Primers	Sequence	Source
UGP2 forward	ATGTCTCAAGATGGTGCTTCTCA	IDT
UGP2 reverse	GGTGTGCTCAAATTCATGTGATG	IDT
UGDH forward	TGCCCAGAGAATAAGCAGCAT	IDT
UGDH reverse	CCATTCCAATCGCTGTTGCTA	IDT
UAP1 forward	AATGACCTCAAACTCACGTTGT	IDT
UAP1 reverse	GCTCTGCATAAAGTTCTACCTGT	IDT
HAS2 forward	GATGCATTGTGAGAGGTTTC	Sigma
HAS2 reverse	CCGTTTGGATAAACTGGTAG	Sigma
Actin forward	GAGCGCGGCTACAGCTT	IDT
Actin reverse	TCCTTAATGTCACGCACGATTT	IDT
TBP forward	GAGCCAAGAGTGAAGAACAGTC	IDT
TBP reverse	GCTCCCCACCATATTCTGAATCT	IDT

Table 9: List of oligonucleotides for shRNAmir cloning (97mer)

97mer oligonucleotides	Sequence	Source
UGP2_1	TGCTGTTGACAGTGAGCGAAGTGTTGATTTTTAAAATAGA TAGTGAAGCCACAGATGTATCTATTTTAAAAATCAACACT GTGCCTACTGCCTCGGA	Invitrogen
UGP2_2	TGCTGTTGACAGTGAGCGAGGCTAGTTTCTTACAATGAAA TAGTGAAGCCACAGATGTATTTCATTGTAAGAAACTAGC CCTGCCTACTGCCTCGGA	Invitrogen

4 - DISCUSSION

In this thesis, I focused on unifying the theme of metabolism and the acquisition of metastatic properties by cancer cells, namely increased migration. I believe to have contributed for this goal with two studies, in distinct settings, where the role of metabolism in the migratory properties of cells is evaluated. Chapters 3.1 and 3.2 provide a detailed discussion of the results obtained in each of those two studies. In this final chapter, I will try to unify our own data with data previously published in the literature and draw the main conclusions.

Migration is a fundamental cellular phenotype that endows cancer cells the ability to disseminate from the primary tumor, a crucial step in metastasis formation. Understanding the metastasis process is of utter importance, since most of cancer-related deaths are directly related with the presence of metastases. In this context and considering the growing evidence pointing to an important role of metabolism in cancer progression, I first concentrated my efforts on understanding how altered metabolism, more specifically OXPHOS dysfunction, has an impact on cellular phenotypes associated with tumorigenesis, namely increased migration. Indeed, we showed that OXPHOS dysfunction *in vitro*, caused a mtDNA mutation, leads to increased migration in tumor cells (chapter 3.1) in a process that seems to mediated by integrin-α5β1. This prompted us to ask if, on the other hand, a more migratory and invasive phenotype is accompanied by a metabolic rewiring. For that, we optimized an EMT model in mammary epithelial cells and we found profound metabolic alterations, affecting multiple pathways, when cells engaged on EMT. We decided to focus our attention on the UDP-glucose pathway, a less-well known glucose destiny that may be crucial for providing regulators of cellular migration (chapter 3.2).

The role of mitochondrial dysfunction was the first proposed cause for the aerobic glycolysis phenotype of cancer cells, by Warburg himself (Warburg 1956). Indeed, nowadays only a few metabolic enzymes have been identified as directly contributing to cell transformation and the most well-known examples are mitochondrial proteins. Mutations in *SDH* have been shown to predispose for neuroendocrine tumors paragangliomas and phaeochromocytomas (Astuti, Douglas, et al. 2001; Astuti, Latif, et al. 2001; Baysal et al. 2000; Burnichon et al. 2010; Gimm et al. 2000; Hao et al. 2009; Niemann and Müller 2000) and we have identified a highly prevalent founder germline *SDHB* mutation in the Portuguese population (Martins and Nunes et al. 2013 - manuscript in annex I). *SDHB* mutations might be of particular relevance for the clinical management of paraganglioma and pheochromocytoma patients, since they have been proposed to be a risk factor for malignancy and poor prognosis (Amar et al. 2007; Gimenez-Roqueplo et al. 2003). Interestingly, SDHB-mutant metastatic PGL/PCC tumors cluster together in an unsupervised hierarchical cluster analysis using microarray data of EMT-related genes and Twist is one of the genes that is overexpressed in this subset of PGL/PCC, when compared with non-metastatic tumors or non-SDHB-metastatic tumors (Loriot et al. 2012). Besides, in PGL/PCC

tumors, Snail/Slug displays a nuclear localization in the SDHB-related metastatic tumors, further supporting the activation of EMT in these tumors (Loriot et al. 2012). In PGL/PCC tumor biopsies, immunohistochemistry staining analysis indicated that Snail may be predictive of the metastatic potential of PCC (Häyry et al. 2009). The association of *SDHB* inactivation with EMT has also been described for other neoplasias, like CRC, HCC and ovarian cancer. In CRC and HCC, lack of expression of SDHB is correlated with advanced tumor staging and *in vitro* experiments showed that with SDHB knock-down cells become more migratory, downregulate E-cadherin and upregulate several mesenchymal associated proteins and EMT TFs like Snail, which is consistent with EMT (Tseng et al. 2018; Wang, Chen, and Wu 2016). In ovarian cancer, loss of SDHB expression leads to a hypermethylated epigenome that is sufficient to promote EMT, characterized by overexpression of Snail and Twist1/2, loss of membrane-bound E-cadherin, together with enhanced ability to form colonies in soft agar (Aspuria et al. 2014). In terms of metabolic phenotype, in HCC cells, SDHB loss also causes increased glucose uptake and lactate secretion while in ovarian cancer, its results in a diversion of glucose towards the pentose phosphate pathway and nucleotide biosynthesis rather than the TCA cycle, to which glutamine makes an increased contribution (Aspuria et al. 2014; Tseng et al. 2018). Interestingly, while in HCC and ovarian cancer cells SDHB loss promotes tumor cell proliferation, in CRC cells it does not affect cell population growth (Aspuria et al. 2014; Tseng et al. 2018; Wang, Chen, and Wu 2016), illustrating the importance of cell context. Concerning mtDNA genes, alterations in the mtDNA have been described in several cancers (Copeland et al. 2002; Fliss et al. 2000; He et al. 2010; Petros et al. 2005), in some instances, like breast cancer, being associated with poor disease-free survival (Tseng et al. 2006).

We contributed to further enlighten the role of mitochondrial dysfunction in cancer cells by showing that a mtDNA mutation that affects all mtDNA-encoded ERC complexes leads to increased tumorigenic and metastatic potential *in vivo* (chapter 3.1). Surprisingly, OXPHOS dysfunction decreased cell growth *in vitro* but led to formation of larger tumors in mice, highlighting the importance of the cellular microenvironment in modulating the consequences of metabolic alterations. Other mice studies have showed a link between mtDNA mutations and the ability to form tumors, like those of Petros *et al*. and Shidara *et al.* (Petros et al. 2005; Shidara et al. 2005) that corroborate our findings. It was also shown that the ability of HeLa cells to form tumors is dependent on the presence of mtDNA (Hayashi, Takemitsu, and Nonaka 1992); however, Kulawiec *et al*. observed that breast cancer cells depleted from mtDNA have higher tumorigenic potential *in vivo* (Kulawiec et al. 2008). Interestingly, it was subsequently demonstrated that stable cell lines derived from primary subcutaneous tumors that grew from ρ0 cells acquired host mtDNA and recovered respiration (Tan et al. 2015). Therefore, it is pertinent

to point out that mitochondrial dysfunction due to mtDNA depletion can be very different from the one caused by mtDNA mutations.

The link between mtDNA mutations and metastasis formation was established when Ishikawa *et al.* showed that the metastatic potential of highly metastatic Lewis lung carcinoma mouse cells accompanies the mtDNA molecules harboring a complex I mutation (Ishikawa et al. 2008). The authors showed that specific mtDNA mutations that induce mitochondrial respiration defects can control the malignant transformation of tumor cells to develop the metastatic potential (Ishikawa et al. 2008); accordingly, the presence of mutated mtDNA is also important for the capacity of breast cancer cells to form metastasis (Imanishi et al. 2011). On the other hand, a recent study highlighted the dynamic nature of metabolism: when cells depleted of mtDNA were injected into mice, the mitochondrial function recovered in a stepwise manner, from cancer cells of the primary tumor, to tumor circulating cells and finally to the cells of the metastasis, suggesting an essential requirement for OXPHOS in tumor progression (Tan et al. 2015).

The higher metastatic capacity conferred by mtDNA mutations has been linked to the production of ROS by dysfunctional mitochondria (Ishikawa et al. 2008; Petros et al. 2005), although in some cases ROS overproduction by metastatic cells does not disappear when the mtDNA mutation is corrected (Imanishi et al. 2011). Another hypothesis is that mtDNA mutation-triggered OXPHOS dysfunction induces a metabolic rewiring that changes the availability of substrates for glycosylation, affecting the abundance of β-1,6 GlcNAc branched N-glycans (Sasai et al. 2002). Accordingly, we detected alterations in the glycosylation pattern of genes that regulate migration, namely integrin β1 (chapter 3.1). The mtDNA-mutated cybrids also showed increased fibronectin expression, a finding already reported in mammary epithelial cells depleted of mtDNA that show enhanced migration and invasion capacity (Kulawiec et al. 2008).

Since most of the cancer metabolism studies are performed under the perspective of proliferation, trying to understand how metabolic reprogramming supports cancer cells' proliferative capacity, much less is known about the metabolic requirements of metastasis. This, together with our results on mtDNA-mutant cybrids, prompted us to investigate possible metabolic alterations that involve the formation of metastasis. For that purpose, we used an EMT model, which is a developmental cellular-biological program that allows cancer cells to acquire features that support their dissemination from the primary tumor site (Brabletz et al. 2018).

We induced EMT in breast epithelial cells by changing ERK2 signaling (chapter 3.2), a pathway that has been shown to be at the crossroads of several EMT inducers (Buonato and Lazzara 2014; Gonzalez and Medici 2014; Xie et al. 2004; Zhang et al. 2012).

ERK signaling through the RAS–RAF–MEK–ERK pathway is an evolutionary conserved signaling cascade that transmits signals from cell surface receptors to promote proliferation and

survival programs (Roux and Blenis 2004). ERK signaling is frequently over-activated in human tumors, usually owing to mutations in RAS or BRAF (Dhillon et al. 2007), while ERK genetic alterations, although very rare, were described in cervical carcinomas (Ojesina et al. 2013). The downstream signaling of ERK is still not fully understood, but ERK signaling seems to be essential for EMT and HGF-induced motility in lung cancer cells (Buonato and Lazzara 2014; Radtke et al. 2013). Depending on the pool of substrates that is activated, ERK can trigger different phenotypes. For example, DEF motif-signaling of ERK2 can have a specific role in EMT, in a process mediated by Fra1 stabilization and ZEB activation (Shin et al. 2010). A newly identified ERK substrate MCRIPT was shown to regulate EMT through E-cadherin (Ichikawa et al. 2018). The regulation of cellular metabolism by ERK is a poorly explored field. The few existing studies revealed that ERK mediates the signaling triggered by ECM attachment and ErbB2, which promotes the entry of glucose in the TCA cycle by upregulation of PDK4 (Grassian et al. 2011). Interestingly, ERK2 activation promotes mitochondria fragmentation through Drp1 phosphorylation, which is required for tumor growth in Ras-expressing cells (Kashatus et al. 2015). The fragmentation of mitochondria via Drp1 is enhanced in invasive breast cancer and it is associated with increased metastatic potential (Zhao et al. 2012).

In chapter 3.2, we showed that EMT induction upon ERK2-DEF motif signaling increased the abundance of UDP-sugar metabolites, especially UDP-glucose. Besides, UGP2, the enzyme that produces UDP-glucose, is not only increased in the cells becoming mesenchymal, but it is actually required for EMT. There are a few metabolomics studies that indicate the increase in UDP-glucose and UGP2 in EMT models (Lucena et al. 2016; Mulvihill et al. 2014), although they do not address the importance of the UDP-glucose pathway for the mesenchymal phenotype, namely for the acquisition of migratory and invasive features. We propose that UDP-glucose and UGP2 may be important for EMT by mediating the production of HA, which is a glycosaminoglycan generated from UDP-glucuronate (derived from UDP-glucose) and from UDP-GlcNAc. In support of our hypothesis, both UDP-glucuronate and UDP-GlcNAc and the enzymes that catalyze their production are increase in our mesenchymal cells (Fig. 13). We demonstrated, for the first time, that ERK2-driven EMT underlies a metabolic reprograming, involving UDP-glucose pathway, and that ERK pathways is coordinating the expression of several enzymes of the UDP-sugar metabolism.

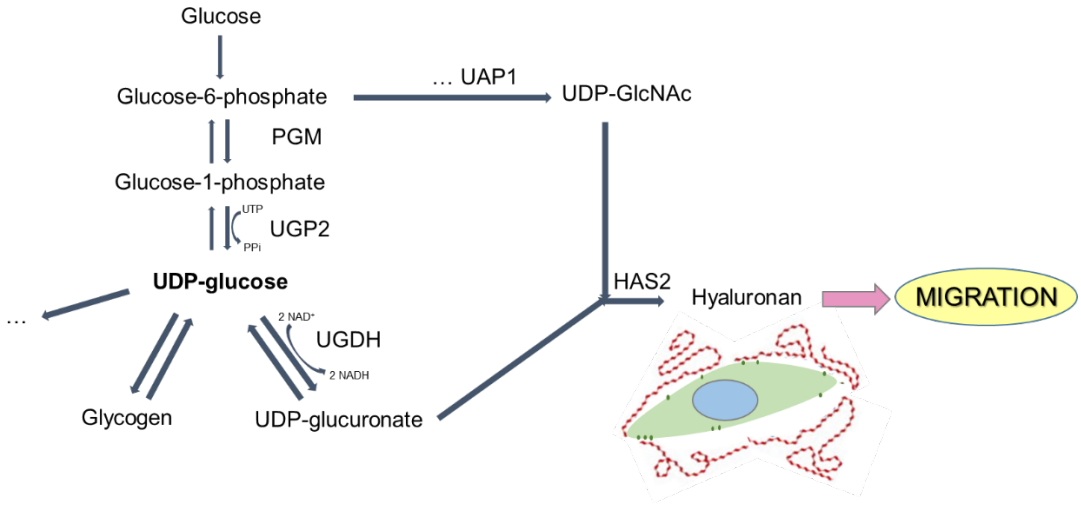

Fig. 13: Proposed model for how is UGP2 and UDP-glucose metabolism regulating EMT-associated migration.

Some papers have already reported metabolic alterations after EMT induction using several methods: treatment with TGF-β, HGF, overexpressing EMT TFs (like Snail) or activating other pathways (such as Wnt or Hippo). Despite the fact that the majority of studies focuses on glucose metabolism, these changes are very diverse and encompass several different pathways, of which the regulation of the production of fructose-1,6-bisphophate from fructose-6-phosphate and the reverse reaction stands out: both PFK platelet (PFKP) and FBP1 are directly regulated by Snail through binding to the respective promoters (Dong et al. 2013; Kim et al. 2017). On the other hand, metabolic alterations can also result in the acquisition of mesenchymal features by epithelial cells. This topic is discussed in more detailed in annex II.

Recently, some studies have been published concerning the metabolism of metastatic cells. For instance, PGC-1α (peroxisome proliferator-activated receptor-γ co-activator 1α), a transcriptional coactivator that regulates the genes involved in energy metabolism, was shown to be essential for migration capacity and metastasis of breast cancer cells (LeBleu et al. 2014). The authors showed that circulating cancer cells originated from breast tumors respire more, display increased levels of mtDNA and have increased PGC-1α expression. The expression of this enzyme, in turn, was shown to determine the *in vitro* invasive capacity of cancer cells. Moreover, suppression of PGC-1α expression suppresses cancer cell dissemination and metastasis *in vivo*, by facilitating intravasation and extravasation of cancer cells (LeBleu et al. 2014). These results are in line with the previously described study where circulating tumor cells derived from tumors that emerged after injection of breast cancer cell line depleted of mtDNA show increased respiration compared to cells from the primary tumor and decreases when compared with cell from the metastasis (Tan et al. 2015). On the other hand, using a xenograft mouse model of melanoma, Luo *et al.* showed that PGC-1α suppression increased metastasis and circulating tumor cells derived from these

tumors had lower PGC-1α expression. The authors suggest that PGC1α has a dual function in cancers: promoting growth and survival of tumors, while suppressing cell motility, cell–cell interaction, adhesion and invasion that promote metastasis formation (Luo et al. 2016). The difference between these two studies concerning PGC-1α role in metastasis may be due to differences in cell of origin: breast cancer cells are of epithelial origin while melanoma cells originate from neural crest cells and are mesenchymal.

In a different study, Piskounova *et al.* studied the metabolism of melanoma cells that are more successful at forming metastasis (denominated by the authors as "efficiently metastasizing melanomas") using patient derived xenografts. These cancer cells are more efficient at forming metastasis due to their increased ability to survive in the blood and in the secondary tumor site (Piskounova et al. 2015). Metastatic tumors originated from the "efficiently metastasizing melanomas", when compared to the subcutaneous tumors, display an altered metabolism that is characterized by an enhanced contribution of glucose carbon to tissue serine and glycine levels and increased expression of folate pathway enzymes (Piskounova et al. 2015). This pathway is a major source of NADPH, an important intermediate that allow cells to cope with oxidative stress and in fact, the authors show that circulating melanoma cells and metastases have elevated ROS compared to primary tumors and that oxidative stress is a limiting factor for the establishment of metastasis (Piskounova et al. 2015).

Together, these studies highlight the active role of mitochondrial function for metastasis formation. Besides, it is crucial that cells adapt their metabolism to cope with the oxidative stress in hostile environments like the circulation and the secondary tumor site. Further studies have highlighted the importance of other metabolic pathways for metastasis formation such as proline or lysine metabolism (Elia et al. 2017; Wu et al. 2015). Importantly, Dupuy *et al.* showed that breast cancer cells show metabolic heterogeneity depending on the metastatic site and that metastatic cancer cells can engage both glycolytic and oxidative metabolism (Dupuy et al. 2015). In fact, breast cancer cells that metastasize to the lung show increased pyruvate carboxylase-dependent anaplerosis, which is modulated by nutrient availability within the lung microenvironment (Christen et al. 2016).

The two studies that compose this thesis were performed *in vitro*, which comes with limitations, the most relevant probably being the culture conditions in which most of the experiments were made. Like in any eukaryote cell, the metabolism of cancer cells is an adaptation of cells to the extracellular conditions, hence being highly influenced by the tumor microenvironment. As such, our results obtained should be interpreted in the light of culture conditions where cells have immediate access to nutrients and are exposed to excess oxygen. Besides, a tumor functions as a collective entity composed of multiple cell types and a complex matrix, similarly to an organ,

which differs greatly from our culture conditions. On the other hand, the simplicity of *in vitro* studies allows for an increased control of the variables and these studies have given us insightful cues about the importance of old and new pathways. Nowadays, a huge effort has been put to finding *in vitro* conditions that, for example in terms of nutrients availability, most appropriately mimic the *in vivo* conditions, as described in Cantor *et al*. and Muir *et al.* (Cantor et al. 2017; Muir et al. 2017). Importantly, the intracellular abundance of the UDP-sugars studied in this thesis is similar when comparing cells cultured in standard media from cells cultured in a media which better reflects the polar metabolite composition of human plasma, as evidence in the article by Cantor *et al.* (Cantor et al. 2017).

In order to understand the metabolic requirements of cancer cells during tumor progression, namely during the acquisition of migratory and invasive properties that enable metastasis, we used an *in vitro* EMT model that consisted in overexpressing ERK2 in MCF10A cells. MCF10A cells are immortalized mammary epithelial cells and although non-transformed, they harbor genetic abnormalities: a deletion of the locus containing p16 and p14ARF, as well as amplification of *Myc* (Debnath, Muthuswamy, and Brugge 2003). This *in vitro* model presents, therefore, limitations but it consists of a good platform to identify and unravel the mechanism of potential new candidates that regulate EMT, like UGP2, and as a first screening system for pharmacological interference with the process. Future studies should, consequently, focus on translating the findings presented here concerning UDP-glucose pathway and UGP2 expression into other models closer to *in vivo* natural systems. MCF10A cells have the advantage of being able to form three-dimensional structures when cultured in a matrix such as Matrigel®, providing an important tool to model early events involved in carcinoma formation (Debnath et al. 2002; Debnath, Muthuswamy, and Brugge 2003). In fact, it has been shown that these cells when overexpressing Ras-V12, form large colonies exhibiting widespread protrusive extensions and disruption of acinar morphogenesis (Shin and Dimitri et al. 2010). Hence, it would be interesting to understand if the formation of these extensions is impaired upon UGP2 silencing. Besides, the analysis of UGP2 expression in a breast cancer cell lines panel and in breast cancer tumor biopsies would allow to understand if it correlates with tumor aggressiveness and patient survival. Additionally, it would be important to look into tumor specimens and evaluate where UGP2 is mainly expressed: if in the center of the tumor or in the invasive front and whether it is associated with the expression of EMT markers. Ultimately, mouse models of breast cancer should be used to understand if UGP2 and the UDP-glucose metabolic pathway are limiting for tumor progression and if they cause liabilities that can be exploited as an innovative approach to manage metastasis.

Altogether, the data presented in this thesis establishes a clear link between metabolic alterations and the migration and invasion capacities of cells, using a model of EMT. For the first time, we

demonstrated that ERK2, a pivotal molecule that coordinates several oncogenic pathways, regulates UDP-sugar metabolism, in this way favoring two major features of metastasis: increased migration and invasion. The observations in this thesis may present useful opportunities in clinical oncology, particularly the use of the metabolic pathways and enzymes as predictive biomarkers or even imaging techniques. Finally, our results support the notion of the dependence on certain types of metabolism as an important target for the development of new drugs to control cancer dissemination and metastasis, which could have a significant impact in decreasing cancer-associated deaths.

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6 - ANNEXES

6.1 - ANNEX I: A Founder SDHB Mutation in Portuguese Paraganglioma Patients

A founder *SDHB* mutation in Portuguese paraganglioma patients

Dear Editor

We would like to report a genetic screening of *SDHB*, *SDHC*, *SDHD* and *SDHAF2* genes (hereafter abbreviated to *SDHx*) in patients with paragangliomas (PGL) and phaeochromocytomas (PCC) from northern Portugal.

PGL and PCC are neuroendocrine tumours that can be caused by heterozygous germline loss-of-function mutations in *SDHx* genes (Gimenez-Roqueplo *et al.* 2012). The spectrum of germline *SDHx* mutations varies considerably among different countries, a circumstance that is related to the existence of several founder mutations. This is particularly marked in The Netherlands (Hensen *et al.* 2012), where six founder mutations were discovered, but evidence for founder effects was also observed for *SDHx* mutations in Austrian (Janecke *et al.* 2010), Italian (Simi *et al.* 2005) and Spanish (Cascon *et al.* 2009) populations. In addition to familial PGL or PCC, germline *SDHx* mutations have also been observed in patients without familial history of disease, sometimes classified as 'occult' familial cases (Lima *et al.* 2007). This phenomenon may be associated with the low penetrance of *SDHx* mutations, which is about 50%, although increasing in older individuals (Burnichon *et al.* 2009), and with the genomic imprinting observed in *SDHD* and *SDHAF2* genes (Gimenez-Roqueplo *et al.* 2012).

We report the genetic study of 37 individuals diagnosed with PGL or PCC between 2009 and 2012, of which three were familial cases and 34 were sporadic. Sporadic cases were defined as those having no familial history of PGL or PCC in the parental and grandparental generations and disease was considered to be inherited when at least two first-degree relatives or two second-degree relatives were affected by these tumours. Only the index cases from familial PGLs were considered for all the analyses. Patients displaying syndromic features associated with VHL, MEN2 or NF1 were excluded from the study. Written informed consent for genetic testing was obtained from all patients.

The majority of patients developed PGL (27/37; 73.0%) and ten developed PCC (27.0%). The clinical and pathological features of the 37 patients are shown in Table 1. We have identified seven different germline *SDHx* mutations that were present in 20 of the 37 (54.1%) patients. One mutation was located in *SDHD* and six were located in the *SDHB* gene, while no alterations were found in *SDHC* or *SDHAF2* genes.

We identified three cases of familial PGL. In the first, the index case (multiple HN PGLs and a thoracic PGL) was found to harbour a germline *SDHD* frameshift mutation (L139FfsX29) that was shared by the two relatives with disease (one with multiple abdominal PGLs and another with multiple head and neck PGLs). Two additional families with PGL were identified: in one, the index case developed a malignant abdominal PGL (with bone metastases) and her uncle had also developed a malignant abdominal PGL with brain metastases; in a third family, the index case presented abdominal PGL and his mother had developed multiple head and neck PGLs. All the affected relatives of these two families harboured a large germline 15 678 bp deletion in the *SDHB* gene, which encompasses the promoter and exon 1.

In the 34 patients in whom no familial relationships could be established (considered as sporadic cases), we have identified six germline *SDHx* mutations, all in *SDHB*, that were present in 17 patients (50.0% of sporadic cases). The majority of the germline-mutated patients harboured the *SDHB* 15 678 bp deletion (9/17, 52.9%), four patients presented *SDHB* frameshift mutations (P56delYfsX5 and S198AfsX22 in three patients) that lead to premature stop codons and four patients presented germline missense mutations (A43P, R11H and R230H in two patients). All the aforementioned *SDHB* mutations were previously described as pathogenic (Bayley *et al.* 2005). In patients without *SDHx* mutations, we have screened for *VHL*, *TMEM127* and *MAX* mutations, but none were disclosed.

The finding that a large number of patients in this series (11/37; 29.7%) presented the germline *SDHB* 15 678 bp deletion prompted us to study the haplotypic variability in the flanking regions upstream (using the SNPs: rs1569754, rs3946080, rs2143811 and rs5772743) and downstream (using the SNPs: rs7545518, rs7545499

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Table 1 Clinical characteristics of PGL and PCC patients with or without *SDHx* mutations

	All patients ($n=37$)	Patients with germline mutations[a] ($n=20$)	Patients without germline mutations ($n=17$)	Mutated vs non-mutated cases *P* value[b]
Mean age at diagnosis (years)	40.0±16.0	33.8±14.6	47.4±14.8	0.008
Gender				
Male	21 (56.8)	14 (70.0)	7 (41.2)	0.078
Female	16 (43.2)	6 (30.0)	10 (58.8)	
Multiple tumours	1 (2.7)	1 (5.0)	0	NS
Mean tumour size (cm)	7.2±3.6	7.1±3.2	7.3±4.1	NS
Localization[c]				
Adrenal glands	10 (27.0)	2 (10.0)	8 (47.1)	0.023
Extra-adrenal				
Head and neck	7 (18.9)	4 (20.0)	3 (17.6)	
Thorax	4 (10.8)	4 (20.0)	0	
Abdomen	17 (45.9)	11 (55.0)	6 (35.3)	
Hormonal hypersecretion[d]				
Norepinephrine	21 (67.7)	12 (75.0)	9 (60.0)	NS
Epinephrine	5 (17.2)	0 (0)	5 (38.5)	0.011
Dopamine	14 (46.7)	7 (43.8)	7 (50.0)	NS
Metanephrine	6 (20.0)	0	6 (35.3)	0.005
Normetanephrine	21 (67.7)	11 (68.8)	10 (66.7)	NS
None	7 (23.3)	3 (18.8)	4 (28.6)	NS
Locally invasive disease without metastases	6 (16.2)	3 (15.0)	3 (17.6)	NS
Distant metastases	11 (29.7)	7 (35.0)	4 (23.5)	NS
Mortality	4 (10.8)	3 (15.0)	1 (5.9)	NS

The numbers in parentheses indicate percentage values.
[a]Only the index cases of familial PGLs are shown.
[b]$P<0.05$ is considered statistically significant.
[c]One familial case had PGL in head, neck and thorax.
[d]Percentages were calculated considering only the patients that had hormonal measurements; hormonal hypersecretion was defined as any value above normal range.

and rs7536679) of the deletion breakpoint, in order to assess whether this deletion occurred in multiple independent events or whether it was a single event that settled in the population. After selective amplification of the deleted allele in the 11 deletion-positive patients, we observed that, in all cases, the deletion was associated with the same haplotype (haplotype 3 – Table 2); selective amplification of the WT alleles (in the same 11 deletion-positive patients) disclosed two different haplotypes. Furthermore, haplotype reconstruction (using the same

Table 2 Haplotypes detected in PGL patients, control population and other European populations

	SNPs							Chromosome frequency (%)				
Haplotype	rs1569754	rs3946080	rs2143811	rs5772743	rs7545518	rs7545499	rs7536679	PGL[a] $n=11$	NPO $n=80$	GBR $n=178$	FIN $n=186$	TSI $n=196$
Hap1	C	A	C	A	A	A	T	0	5.0	0	0	0
Hap2	C	A	C	A	G	G	C	0	22.5	28.7	29.6	29.6
Hap3	**C**	**A**	**C**	**–**[b]	**A**	**A**	**T**	**100**	**45.0**	**52.3**	**54.8**	**47.5**
Hap4	C	A	C	–[b]	G	G	C	0	13.8	7.3	8.6	8.2
Hap5	T	G	T	A	A	A	T	0	1.3	0	0	0
Hap6	T	G	T	A	G	G	C	0	12.5	11.8	7.0	14.8

NPO, North of Portugal (control population); GBR, British in England and Scotland (1000 Genomes Project population); FIN, Finnish in Finland (1000 Genomes Project population); TSI, Toscani in Italia (1000 Genomes Project population); Hap3, given in bold, indicates the haplotype found in all deleted alleles.
[a]PGL, includes only the deleted alleles from the 11 patients harbouring the SDHB 15 678 bp deletion.
[b]Dash indicates adenine deletion.

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SNPs) in 40 healthy individuals from northern Portugal (in whom we confirmed the absence of the deletion) identified four additional haplotypes (Table 2). Four of these haplotypes were detected in other European populations (Great Britain, Finland and Italy) in comparable frequencies (Table 2). The frequency of haplotype 3 in the deleted chromosomes is significantly different from its frequency in the control population ($P=0.0005$).

SDHA and SDHB immunohistochemistry in the tumour tissue showed that all patients with *SDHx* mutations had loss of SDHB and presence of SDHA expression in the tumour. On the other hand, patients without *SDHx* mutations presented both SDHB and SDHA expression, discarding the existence of additional *SDHx* alterations, such as large deletions, that would be missed by Sanger sequencing.

When performing genotype–phenotype correlations, we observed that patients with *SDHx* mutations had a lower mean age at diagnosis ($P=0.008$), developed tumours that were more frequently located in extra-adrenal sites ($P=0.023$) and were predominantly men ($P=0.078$) (Table 1). The tumours that occurred in patients with *SDHx* mutations presented a signature of low secretion of epinephrine ($P=0.011$) and metanephrine ($P=0.005$) (Table 1) when compared with tumours without *SDHx* mutations. Malignant and lethal tumours were more frequently observed in patients with *SDHx* mutations (35.0 and 15.0% respectively) than in non-mutated cases (23.5 and 5.9% respectively), although the difference was not statistically significant.

In the series of PGL/PCC presented here, half of the patients without familial history of disease (17/34; 50.0%) harboured germline *SDHx* mutations; altogether, germline *SDHx* mutations were the primary cause of PGL and PCC in 54.1% (20/37) patients.

The prevalence of *SDHx* mutations in this cohort is high and comparable to the one found in a large series of French PGL patients (Burnichon *et al.* 2009). The high frequency of *SDHx* mutations in our series is likely attributable to the prevalence of the *SDHB* 15 678 bp deletion (55.0% of the *SDHx*-mutated patients). To the best of our knowledge, this is only the second report of the *SDHB* 15 678 bp deletion in PGL/PCC patients, despite the large number of studies in this topic, raising the hypothesis that the high frequency of this deletion in Portuguese patients can be related to a founder effect. A founder effect for this specific deletion had already been proposed by Cascon *et al.* (2008), who studied five families, all of them harbouring this deletion and showing a conserved breakpoint. Notably, of the five families, one was from Porto, Portugal, a second was a Brazilian family and the remaining three were of Spanish origin, more specifically from Galicia (Cascon *et al.* 2008).

Our results show that the deletion breakpoint is conserved among 11 patients with PGL/PCC and also matches the previously reported breakpoint (Cascon *et al.* 2008). None of these 11 patients have familial relationships, but all come from northern Portugal. Furthermore, we have shown that all deleted alleles share a common haplotype (haplotype 3), in a significantly higher frequency than in control, healthy individuals (45.0%). The lack of variation in the up- and downstream flanking regions of the deleted allele (in contrast to the normal alleles from the control population, where six different haplotypes were found) fits with a founder effect for this deletion, which has probably settled in the northern Portuguese/Galician populations. A recent study of *SDHx* mutations in Portuguese PGL/PCC patients from central and southern Portugal did not find this deletion (Domingues *et al.* 2012), further suggesting that the founder effect is related to northern Portuguese/Galician populations.

Our results support the previously reported association between *SDHx* mutations and lower age at diagnosis and extra-adrenal location (Burnichon *et al.* 2009). The association between absence of *SDHx* mutations and higher levels of epinephrine and metanephrine hypersecretion may reflect the higher proportion of PCC in this subgroup of patients, as epinephrine is synthesized and stored only in the adrenal medulla (and metanephrine is its main metabolite) whereas norepinephrine and dopamine are also synthesized in the peripheral sympathetic nerves.

Summing up, we present data showing that the majority of PGL in northern Portugal patients develop as a consequence of germline *SDHx* mutations, in particular a founder mutation in the *SDHB* gene (15 678 bp deletion). Our results support the suggestion by Hensen *et al.* (2012) that population specificities, regarding the presence of founder germline *SDHx* mutations, should be taken into account whenever deciding about the genetic testing in PGL/PCC patients.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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6.2 - ANNEX II: Fueling EMT: How Do Cancer Cells Shape Their Metabolism?

Fueling EMT: How Do Cancer Cells Shape Their Metabolism?

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Abstract

The epithelial to mesenchymal transition (EMT) program can be reactivated during metastasis, especially in its first step, when tumor cells detach from the primary tumor, invade the adjacent tissue and intravasate into the vascular system. EMT confers tumor cells the ability to migrate and invade, having been proposed to be one of the cell-biological programs that enables dissemination. Recent research suggests that cellular metabolism is reprogrammed during EMT. In fact, different metabolic pathways, from glycolysis, the TCA cycle and oxidative phosphorylation to hexosamine biosynthetic pathway and lipid metabolism are rewired and contribute to EMT. However, the variety of EMT inducers, read-outs and cell phenotypes make it difficult to understand if there are key metabolic pathways that drive and maintain the mesenchymal phenotype. Here, we tried to build a clearer picture of the metabolism during EMT, according to the signaling pathways involved. There is a lot of enthusiasm in unraveling of the link between metabolism and EMT, although the number of mechanistic studies is still very limited. With this review, we hope to integrate enough evidence to highlight the role of metabolism in the signaling landscape of EMT.

METABOLIC REPROGRAMMING IN CANCER

The metabolism of tumors has been subject of study since the beginning of the twentieth century. The pioneer in the field, Otto Warburg, described a high consumption of glucose and release of lactate by malignant cells, when compared with non-neoplastic cells, even in the presence of oxygen (Warburg 1924). This phenomenon is known as aerobic glycolysis or the “Warburg effect”. Warburg hypothesized that this aerobic glycolysis was irreversible and due to an

impairment of mitochondrial respiration (Warburg 1956). Nowadays, with an exponential interest in this field of research and the inclusion of the "reprogramming of energy metabolism" as a hallmark of cancer (Hanahan and Weinberg 2011), a more complex picture has emerged.

The adaptation of the cellular metabolism is very important in the sense that it allows cells to maintain cellular homeostasis and survive under different environmental conditions, being part of the adjustment to a phenotypic alteration. Therefore, during malignant transformation and tumor progression, cancer cells rely on specific changes in metabolic activity in order to meet their energetic demands and biomass production. Nowadays, we know that these changes are not limited to carbohydrate metabolism but they also comprise lipids, nucleotides, amino acids, as well as alterations in reductive equivalents and redox power. Besides, different types of tumors display different metabolic adaptations and even within the tumor microenvironment there is metabolic cooperation between cancer cells and stromal cells. The role of metabolism in tumorigenesis is also linked to other cancer-related aspects, such as stemness, angiogenesis, immune escape, among other. Importantly, cancer cell metabolism reflects the stage of the tumor, implying that it may vary according to the proliferation rate and to the migratory and invasive capacities of cancer cells. Indeed, much less is known about the metabolism of migratory cancer cells and of those that are detaching from the primary tumor, as the first step of metastasis.

Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) is a reversible process by which epithelial cells acquire mesenchymal properties. It was initially characterized during embryonic development and it plays a crucial role during gastrulation and neural crest formation. Additionally, it has also been described in other contexts such as wound-healing, fibrosis and cancer (Thiery et al. 2009). During cancer progression, EMT is one of the important mechanisms enabling dissemination of cells from the primary tumor towards the formation of secondary tumor sites, a process known as metastasis (Thiery et al. 2009). Although the contribution of EMT for metastasis is still matter of debate (Fischer et al. 2015; Zheng et al. 2015), EMT can be viewed as part of the more general concept of cell plasticity, which allows cancer cells to adapt and endure different environmental settings, being crucial for cancer progression (Nieto 2013).

Cancer-related EMT is characterized by alterations in cell morphology, namely in the apico-basal polarity and cell-cell adhesion, in the ability to modulate the extracellular microenvironment, by increased capacity to migrate and invade and increased survival. Moreover, EMT is sometimes accompanied by changes in cell proliferation and also in the stemness of cancer cells (Thiery et al. 2009). Such a distinct phenotype naturally reflects the existence of global alterations inside the cells, including dramatic changes in gene expression as well as cellular metabolism.

The most common features of EMT are changes in cell morphology and increased migration and invasion. Epithelial cells display an apical basal polarization and usually form monolayers, contacting with the neighboring cell through cell-to-cell junctions. During EMT, cell junctions undergo re-structuring, reflecting changes in the expression and localization of the proteins that form them. One such protein is E-cadherin, a main component of adherens junctions encoded by the *CDH1* gene, whose expression is frequently suppressed in cancer and associated with the loss of the epithelial phenotype (Batlle et al. 2000; Cano et al. 2000; Grooteclaes and Frisch 2000; Petrova, Schecterson, and Gumbiner 2016). Additionally, post-translational modifications control the activity and localization of E-cadherin during EMT (Fujita et al. 2002; Pinho et al. 2011). Zonula occludens-1 (ZO-1) is a protein also expressed in epithelial tissues, forming tight junctions at the membrane, whose loss of functional activity has been associated with EMT (Huang, Guilford, and Thiery 2012). On the other hand, EMT is characterized by the upregulation of another type of cadherin - N-cadherin - which is associated with an invasive phenotype (Hazan et al. 2000; Islam et al. 1996). The elongated shape and migratory and invasive phenotype of cells undergoing EMT, also reflect changes in the cytoskeleton, for example, the upregulation of the intermediate filament vimentin (Mendez, Kojima, and Goldman 2010). In addition to cellular components, there are also important modifications in the extracellular microenvironment, which becomes more permissive to cellular migration and invasion. This happens with the expression of proteins that modulate the extracellular matrix, regulate cell adhesion and mediate the interaction of cells with the microenvironment, such as fibronectin, metalloproteases and integrins (Park and Schwarzbauer 2014; Yu and Stamenkovic 2000). The expression and the localization of the aforementioned proteins that characterize an epithelial or a more mesenchymal state are used as markers of EMT.

EMT is orchestrated by transcription factors that translate the signals from the microenvironment to specific intracellular pathways. These so called EMT transcription factors (EMT TFs) belong to three main families – Snail, Twist and Zeb - and constitute a complex interactome (Nieto et al. 2016). Snail and Slug (in humans SNAI1 and SNAI2, respectively) belong to the Snail superfamily of zinc-finger transcription factors, while Twist (TWIST1 in humans) is a basic helix–loop–helix transcription factor, a family that includes several master regulators of lineage specification and differentiation (Barnes and Firulli 2009; Manzanares, Locascio, and Nieto 2001; Simpson 1983). The zinc-finger E-box-binding (ZEB) transcription factors consists of two members in vertebrates, ZEB1 and ZEB2 (or SIP1), which bind regulatory gene sequences at E-boxes to repress or activate transcription (Vandewalle, Van Roy, and Berx 2009). These EMT TFs target crucial regulators of EMT, such as E-cadherin, which can be repressed by Snail (Batlle et al. 2000; Cano et al. 2000), Slug (Bolós et al. 2003; Hajra, Chen, and Fearon 2002), Twist (Vesuna et al. 2008; Yang et al. 2010) and Zeb1/2 (Comijn et al. 2001; Grooteclaes and Frisch

2000). Moreover, these transcription factors also lead to the upregulation of key mesenchymal proteins: for example, Snail regulates the expression of integrin-αV and induces migration (Haraguchi et al. 2008) and Twist upregulates N-cadherin through binding to the first intron in prostate cancer cells (Alexander et al. 2006). In the last years, several studies uncovered a complex regulatory network of EMT, not only at the transcriptional level but also in the epigenome, alternative splicing and post translational modifications, all of which modulate the stability, activity and localization of various EMT-related proteins (Nieto, Huang, Jackson and Thiery. 2016). For example, the activity of EMT TFs is also regulated by post translational modifications mediated by know players in tumorigenesis (Zhang et al. 2012; Zhou et al. 2004).

EMT can be induced by a variety of extracellular agonists, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor β (TGF-β), Wnt and Notch (Thiery et al. 2009). Other tumor extracellular signals also induce Snail expression and/or stabilization, like the availability of nutrients and oxygen or signals from other cells in the tumor microenvironment. As an example is hypoxia that results in Snail upregulation, together with a decreased E-cadherin and β-catenin expression (Imai et al. 2003). Moreover, macrophages produce inflammatory cytokines like interleukin-1β (IL-1β) that has been associated with progression of head and neck squamous cell carcinoma (HNSCC) and promotes Snail upregulation and Snail-mediated *CDH1* downregulation. Interestingly, in colon cancer cells, IL-1β ability to stabilize Snail is dependent on the Wnt pathway (Kaler, Augenlicht, and Klampfer 2012). These various agonists, TFs and cellular systems have been utilized by researchers to help defining the molecular mechanisms associated with EMT.

EMT AND METABOLISM

Metabolic Reprogramming during EMT

There are several studies that show a metabolism re-wiring during EMT. This is conceivable since metabolism is highly dynamic and crucial for the adaptation to a new cellular phenotype with distinct needs in terms of nutrient demands for anabolism and energy production.

Snail is one of the EMT effectors where wiring with metabolism has been shown in greater detail. In a microarray analysis performed in colorectal cancer (CRC) cells and in breast epithelial cells, Snail overexpression resulted in differential expression of enzymes from several metabolic pathways (Bhowmik et al. 2015; De Craene, van Roy, and Berx 2005). Moreover, using a metabolomics approach, Snail was shown to induce a metabolic reprogramming in breast epithelial and breast cancer cells (Bhowmik et al. 2015; Kim et al. 2017). In the case of Twist, its role in regulating metabolism during EMT is less well studied than the role played by Snail, but

its overexpression in human mammary epithelial cells also caused alterations in the cellular metabolic profile (Bhowmik et al. 2015).

The metabolic changes induced by TGF-β seem to be highly dependent on the cellular context. This is probably not dissociated from the fact that TGF-β has pleiotropic effects on cells (Massagué 2008) and that several transcription factors, such as Snail, Slug or Zeb, have been shown to directly mediate some of the TGF-β-related metabolic changes. Soukupova *et al.* analyzed the metabolic profile of hepatocellular carcinoma (HCC) cells upon TGF-β modulation (either treating cells with TGF-β or inhibiting the pathway) and revealed a number of metabolites whose abundance was altered (Soukupova et al. 2017). Similarly, when transformation and malignant progression are induced in mammary epithelial cells through RAS and the Hippo pathway, with a concomitant EMT, there is reprogramming of the metabolism of cells as evidenced by alterations in metabolite levels and differential expression of metabolic enzymes (Mulvihill et al. 2014).

Other EMT models also show a metabolic reprogramming. For example, Halldorsson and Rohatgi *et al.* revealed interesting differences in metabolism following EMT when building a genome scale metabolic model of a breast epithelial cell line and its mesenchymal counterpart, following three-dimensional (3D) culture conditions (Halldorsson and and Rohatgi et al. 2017). The clustering of tumors according to the expression levels of metabolic genes revealed a tumor cluster with a "mesenchymal metabolic signature" (Shaul et al. 2014). The genes that are required for Twist-induced mesenchymal cell survival and are shared with the "mesenchymal metabolic signature" include several enzymes important for lipid signaling, glycan metabolism and glutathione associated redox regulation (Shaul et al. 2014).

On the other hand, the alterations in metabolic enzymes and metabolic pathways are also linked to EMT. This is the case of several metabolic enzymes that are mutated or whose expression is dysregulated in cancer such as isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH) and fumarate hydratase (FH) (Colvin et al. 2016; Grassian et al. 2012; Sciacovelli et al. 2016; Tseng et al. 2018; Wang, Chen, and Wu 2016). These models, directly implicate metabolic dysregulation with cancer development and also with metastasis.

Metabolic Pathways Altered during EMT

Glycolysis and Lactate Production

The literature concerning the cellular metabolism during EMT focuses on the carbohydrate metabolism, and particularly glycolysis. Glycolysis is a central cellular metabolic pathway that occurs in the cytoplasm. The catabolism of glucose into pyruvate produces intermediate

metabolites for several anabolic reactions and generates 2 ATP molecules (per molecule of glucose). The glycolytic pyruvate can be further oxidized in the mitochondria in the tricarboxylic acid (TCA) cycle where ATP production is maximized. Alternatively, pyruvate can be converted into lactate in the cytoplasm, with the regeneration of NAD^+ from NADH. An increased glucose uptake has been reported by several studies in cells undergoing EMT triggered by commonly used EMT inducers like TGF-β treatment and Snail or Twist overexpression (Kim et al. 2017; Lee et al. 2012, 2015; Li et al. 2010; Liu et al. 2016; Lucena and Carvalho-Cruz et al. 2016; Teo et al. 2017; Yu et al. 2017).

The demonstration of major metabolic changes during EMT makes it crucial to identify the metabolic enzymes that are responsible for wiring EMT signaling with metabolism. One of the most crucial points of regulation occurs during the interconversion of fructose 1,6-biphosphate and fructose-6-phosphate by phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FBP), which belong to glycolysis and gluconeogenesis, respectively. In luminal breast cancer and gastric cancer cell lines, Snail was shown to inhibit the expression of FBP1, a rate-limiting enzyme in gluconeogenesis that limits the glycolytic rate (Dong et al. 2013; Yu et al. 2017). In the case of gastric cancer cell lines, FBP1 downregulation is important for the Snail-induced increase in glucose uptake and lactate production, since these could be reversed by re-expressing FBP1 (Yu et al. 2017). Furthermore, in MCF-7 and T47D luminal cell lines the Snail-induced E-cadherin downregulation can be reversed when the downregulation of FBP1 is corrected. Importantly, Snail was shown to directly bind to the FBP1 promoter, probably causing its downregulation through the formation of a complex with the histone methyltransferase G9A and concomitant increase in H3K9me2 and decrease in H3K9ac (Dong et al. 2013). This hypothesis is strengthened by the observations that Snail and FBP1 expressions are inversely correlated in gastric cancer and in triple negative breast cancer patient samples (Dong et al. 2013; Yu et al. 2017). The aforementioned findings suggest that one mechanism by which Snail increases glycolysis and impacts EMT features (E-cadherin downregulation) is through downregulation of FBP1. On the other hand, Kim *et al*. showed, in a breast cancer model, that a metabolic reprogramming associated with Snail knock-down was linked with an overexpression and increased kinase activity of the glycolysis enzyme PFK, platelet (PFKP), a PFK-1 isoform which produces fructose 1,6-biphosphate from fructose-6-phosphate (Kim et al. 2017). Moreover, the impact of Snail silencing – metabolic phenotype, increased cell death, decreased colony formation and decreased metastasis formation – was dependent on PFKP expression. The relationship between Snail and PFKP seems to be through direct binding of Snail to the PFKP promoter, thus regulating its expression (Kim et al. 2017). Interestingly, the downregulation of FBP (in this case FBP2) and the downregulation and binding of Snail to PFKP promoter was also described in a CRC model of Snail overexpression (De Craene et al. 2005).

These four studies (De Craene et al. 2005; Dong et al. 2013; Kim et al. 2017; Yu et al. 2017) demonstrate an active role of Snail in promoting a metabolic reprogramming, in the case by regulating glycolysis through binding and regulating the promoter of key glycolysis and gluconeogenesis enzymes. It would be interesting to understand which are the relevant aspects of the cellular context, namely the type of signaling that characterizes each cell system, that might underlie the selective binding of Snail to the promoters of FBP1 and PFKP enzymes. Noteworthy, mammosphere-induced EMT also results in FBP1 downregulation in breast cancer cells. In these cells, ZEB1, ZEB2 and Slug are upregulated (Kondaveeti, Guttilla Reed, and White 2015), but it remains to be elucidated if Snail is also upregulated and regulating the expression of these enzymes in this EMT model.

It is important to point out that EMT is frequently associated with decreased proliferation (Mejlvang et al. 2007; Salt, Bandyopadhyay, and McCormick 2014; Turner et al. 2006; Vega et al. 2004), while increased glucose uptake is often associated with proliferation (Vander Heiden, Cantley, and Thompson 2009). Therefore, in the context of EMT, it is also important to understand the flux of glucose, i.e. into which metabolic pathways are the glucose carbons being directed to.

The fate of glucose seems to depend on the EMT inducer and on the cell type. Lactate is fundamental in driving extracellular acidification, which, in turn, is important for promoting cancer cell migration and invasion, as well as improving cancer cell evasion of the immune system (Busco et al. 2010; Calcinotto et al. 2012; Kato et al. 1992; Rozhin et al. 1994). Several studies have reported an enhanced production of lactate in EMT models, such as in Panc-1 pancreatic cancer cells (Liu et al. 2016), MCF-7 breast cancer cells (Lee et al. 2012, 2015) upon TGF-β treatment and also of gastric cancer cells after Snail overexpression (Yu et al. 2017). However, in the case of the breast cancer cell line MDA-MB-231, which display mesenchymal features, two independent studies have reached different conclusions when measuring lactate production upon Snail knock-down: one reports an increased lactate production and the other decreased (Kim et al. 2017; Lee et al. 2012). Such differences may be attributed to distinct culture and experimental conditions, and knock-down efficiency.

Mammosphere culture has also been used to induce EMT and analyze metabolism but two distinct studies in breast cell lines reported different observations concerning glycolysis and lactate secretion. Kondaveeti, Guttilla Reed and White used BT-474 and MCF-7, two breast cancer cell lines, and saw a higher glucose consumption and lactate production in mesenchymal cells (Kondaveeti, Guttilla Reed, and White 2015). In the same model, cells undergoing EMT upregulate glucose transporters (GLUT) (specifically GLUT3 and GLUT12) and enzymes related to lactate production and export (lactate dehydrogenase [LDH] B and monocarboxylase

transporters [MCT] 2 and 4, respectively) (Kondaveeti, Guttilla Reed, and White 2015). On the other hand, Halldorsson and Rohatgi *et al.* observed that the breast non-tumorigenic epithelial D492 cells rely more on glycolysis since they display higher glucose consumption and lactate secretion rates, when compared with the more mesenchymal counterparts that were spontaneously-derived from 3D co-culture with endothelial cells. The epithelial cells also have a higher extracellular acidification rate, a phenomenon commonly correlated with lactate secretion (Halldorsson and Rohatg et al. 2017). This may be explained by the fact that the cell lines used are different and BT-474 and MCF-7 cells after undergoing EMT proliferate more, while D492 cells proliferate less compared to the mesenchymal counterpart (Halldorsson et al. 2017; Kondaveeti, Guttilla Reed, and White 2015).

Hexosamine Biosynthetic Pathway and Pentose Phosphate Pathway

Besides lactate, glucose may also be supporting other metabolic pathways. This is the case of the hexosamine biosynthetic pathway (HBP), which is crucial for substrate production and post-translational protein modifications (Lucena and Carvalho-Cruz et al. 2016). Glycosylation is important for the function of proteins with key roles in EMT, such as Snail (reviewed in Taparra, Tran, and Zachara 2016). Snail can undergo a O-GlcNAc modification at serine 112, which prevents its degradation, thus leading to decreased activity of the E-cadherin promoter. This can be reversed by silencing O-GlcNAc transferase (OGT), one of the enzymes that adds O-GlcNAc, and is increased with higher glucose concentration in the cell culture media (Park et al. 2010). HBP thus seems to play an important role in Snail regulation of EMT, which is further suggested by the finding that OGT overexpression increases migration and invasion (Park et al. 2010). On the other hand, Snail regulates glutaminases (Haraguchi et al. 2013) which have been shown to alter the activity of GFPT1 and the O-glycosylation status of specific enzymes (Donadio et al. 2008).

Pentose phosphate pathway (PPP) originates from the phosphorylated form of glucose, glucose 6-phophate, and generates reducing equivalents and intermediates important for the synthesis of lipids and nucleotides and for antioxidant responses, being, therefore, crucial in sustaining cell survival and proliferation (Stincone et al. 2015). Twist overexpression in non-transformed mammary epithelial cells (MCF10A) results in the upregulation of the rate limiting enzyme of the oxidative branch of PPP glucose-6-phosphate dehydrogenase (G6PD), together with an increase in glucose consumption (Yang and Hou et al. 2015). Despite the fact that cell proliferation was not analyzed, other studies showed that Twist promotes proliferation: Twist knock-down induces a G1 arrest and decreases proliferation in prostate, breast and in gastric cancer cells (Qian et al. 2013; Shiota et al. 2008) and Twist overrides oncogene-induced senescence (Ansieau et al. 2008).

On the other hand, mammosphere induced EMT leads to a downregulation of G6PD and the PPP enzymes from the non-oxidative branch transketolase and transaldolase. Surprisingly, the cells with a more mesenchymal phenotype proliferate more (Kondaveeti, Guttilla Reed, and White 2015). The downregulation of these enzymes may reflect a decreased flux of glucose towards PPP which, as suggested by the authors, may point to a decreased need for redox control mediated by the intermediates of PPP due to a reduced dependency on oxidative phosphorylation (OXPHOS) which generates ROS (Kondaveeti, Guttilla Reed, and White 2015). To complement this hypothesis, it would be interesting to analyze the mitochondria metabolic function in these cells.

Mitochondrial Metabolism: TCA Cycle, OXPHOS and Glutamine Metabolism

Several mitochondrial metabolic reactions have been studied in the context of EMT. They include the tricarboxylic acid (TCA) cycle (also called the Krebs cycle), where carbon fuels like carbohydrates, fatty acids and amino acids are oxidized and the OXPHOS, where high energy electrons are donated to the electron transport chain (ETC) to generate a proton gradient across the membrane that culminates in the generation of ATP and concomitant transfer of electrons to oxygen. The TCA cycle can also produce precursors for the generation of lipids, proteins modifications or signaling molecules. Some reactions can replenish components of the TCA cycle, such as those that produce α-ketoglutarate from glutamine and glutamate. In the context of cancer, increased glycolysis is frequently associated with decreased respiration (Vander Heiden, Cantley, and Thompson 2009). This has been described in EMT models like pancreatic cancer cells treated with TGF-β (Ji et al. 2016), breast cancer cells overexpressing Snail or inducing Wnt signaling (Lee et al. 2012) and in kidney cells overexpressing Snail (Haraguchi et al. 2013). Conversely, in non-small cell lung cancer (NSCLC), the carbon from glucose can also flux through the TCA cycle and lead to increased respiration in TGF-β- and Snail-induced EMT (Jiang et al. 2015; Yuting Sun et al. 2014), although contradictory results have also been reported using TGF-β (Ulanet et al. 2014).

The fate of pyruvate is central to the kind of metabolism that is driving the cell. Through the key enzyme pyruvate dehydrogenase (PDH) in the mitochondria, pyruvate can be converted into acetyl CoA that feeds the TCA cycle. Snail is able to regulate this enzyme that acts as a metabolic hub inside the cell: indeed, Snail overexpression in MDCK cells results in reduced activity of PDH, through the increased promoter activity and consequent mRNA upregulation of its negative regulator, pyruvate dehydrogenase kinase (PDK) 1 (Haraguchi et al. 2013). Concerning the TCA cycle, the enzymes IDH2, aconitase 2, SDH all have decreased activity after Snail overexpression in MDCK cells, although their expression remains unaltered (Haraguchi et al. 2013), suggesting that Snail can regulate the mitochondrial enzymes activity at the at the post-translational level.

Moreover, complexes II and IV from OXPHOS also present a diminished activity, which is in accordance with the reduced ATP content, mitochondrial respiration and mitochondrial membrane potential observed in these cells (Haraguchi et al. 2013). In summary, Snail modulates the mitochondrial metabolism in kidney cells by regulating either the expression or the activity of metabolic enzymes.

Snail and TGF-β signaling are able to regulate the conversion of glutamine to glutamate. Glutamine is commonly used by cancer cells to support their rapid growth and proliferation (DeBerardinis et al. 2007). Glutamine can be converted directly into glutamate by glutaminase (GLS), which in turn can produce α-KG by several enzymes such as glutamate dehydrogenase (GDH) or transaminases. The expression of GLS1 is increased in cells with activated TGF-β signaling, a finding that will be discussed in the TGF-β subsequent section (Lee et al. 2016; Soukupova et al. 2017). Additionally, there seems to be a reciprocal regulation between GLS2 and Snail. Not only GLS2 is downregulated after Snail overexpression in normal kidney cells (Haraguchi et al. 2013) but it also represses Snail expression in HCC cells, which results in decreased motility and metastasis (Kuo et al. 2016). This downregulation occurs at the translational level and is mediated by the binding and stabilization of Dicer, possibly through the proteasome, which promotes miR-34 maturation. The GLS2-Dicer binding occurs in an enzymatic activity-independent manner although further studies should clarify the impact of glutamine metabolism (Kuo et al. 2016). GLS2 expression is inversely correlated with advanced-stage, vascular invasion, early recurrence and poor prognosis in HCC patients (Kuo et al. 2016). Finally, glutamate dehydrogenase 1 (GDH1), which converts glutamate to α-ketoglutarate, is upregulated after Twist expression in MCF10A cells (Farris et al. 2016).

Lipid Metabolism

Some studies explored how lipid metabolism is affected during EMT induced by TGF-β and Snail (Jiang et al. 2015; Yang et al. 2016). Particularly, it has been described that EMT affects the levels of fatty acid synthase (FASN), the enzyme that uses acetyl-CoA and malonyl-CoA to produce palmitate (Jiang et al. 2015; Yang et al. 2016) and the synthesis of ceramides (Edmond et al. 2015). Interestingly, the synthesis of sphingolipids and glycosphingolipids has also been explored during EMT (Guan et al. 2010; Guan, Handa, and Hakomori 2009; Mathow et al. 2015).

SIGNALING PATHWAYS AND EMT

The TGF-β, Wnt and Hippo signaling pathways are crucial in the maintenance of cellular homeostasis, while also playing a role in cancer progression. In this section, we will describe the alterations in cellular metabolism taking place during EMT mediated by TGF-β, Wnt and Hippo pathways.

TGF-β

The TGF-β signaling has an especially prominent role in regulating EMT. The binding of TGF-β and related ligands to the respective receptors leads to the phosphorylation of serine and/or threonine residues (more rarely tyrosine) in their cytoplasmic domain, which results in transduction of the signal to the nucleus. This is mediated by a group of intracellular signaling molecules, called SMAD proteins, or by other signaling pathways such as mitogen-activated protein kinase (MAPK) signaling, thereby initiating a program of gene expression that regulate growth, apoptosis, adhesion, migration and differentiation (Wu and Hill 2009). TGF-β treatment activates several intracellular effectors involved in the EMT phenotype such as Snail (Peinado, Quintanilla, and Cano 2003). The ligand TGF-β itself has 3 variants and all have been described as EMT inducers, being TGF-β1 is the most commonly used in the literature related to the metabolism of EMT (Xu, Lamouille, and Derynck 2009). TGF-β also has an important role in cancer, namely in EMT associated with malignant progression *in vivo* (Cui et al. 1996). This cytokine has been shown to be produced by several tumors, either by the tumor cells themselves or by the cells that constitute the tumor stroma. However, TGF-β effects on tumorigenesis and cancer progression can be very complex and depend on intrinsic characteristics of the cells that are receiving the signal. TGF-β has been shown to promote homeostasis and suppress tumor progression in a premalignant stage, while cancer cells commonly use TGF-β signaling in their favor to undergo EMT, invade the surrounding tissues, evade the immune response and ultimately promote metastatic dissemination and the colonization of secondary tumor sites (Massagué 2008).

In general, cells undergoing EMT upon treatment with TGF-β show an increased glucose uptake. This has been described in lung cancer (Lucena and Carvalho-Cruz et al. 2016), pancreatic cancer (Liu et al. 2016), breast cancer (Lee et al. 2015; Li et al. 2010) and gastric cancer cells lines (Teo et al. 2017). In MCF-7 cells, a cell line generated from a breast cancer metastasis with an epithelial morphology, TGF-β treatment leads to an EMT phenotype characterized by morphological changes, upregulation of vimentin, downregulation of E-cadherin and increased migration. In addition to several EMT features, TGF-β triggers metabolic alterations that affect several

pathways. In fact, MCF-7 cells respond to TGF-β by upregulating GLUT1, increasing the glucose uptake (Li et al. 2010) and also secreting more lactate to the medium (Lee et al. 2015).

TGF-β not only seems to regulate the glucose uptake in cells undergoing EMT but also the way that glucose is metabolized. When comparing two HCC cell lines that express E-cadherin, vimentin and TGF-β at different levels, the cells associated with a more mesenchymal phenotype show an upregulation of PPP enzymes G6PD and transketolase. TGF-β receptor I silencing in HCC cells expressing TGF-β and associated with a more mesenchymal phenotype results in decreased expression of G6PD, its paralog hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase and also 6-phosphogluconolactonase (Soukupova et al. 2017). The authors suggest that the higher expression of the enzymes, together with the lower levels of some intermediates of this pathway observed in the mesenchymal cells, can result from a higher shunt to the PPP. However, according to the authors, these differences in PPP did not correlate with differences in proliferation, but with a switch from fatty acid oxidation to synthesis (Soukupova et al. 2017). Indeed, PPP not only provides intermediates for nucleotide synthesis that are crucial in sustaining cancer cell proliferation but also generates reducing equivalents and intermediates important for the synthesis of lipids and for redox control. So, increased or decreased PPP may be associated with other cellular needs besides proliferation like ROS control. On the other hand, in lung cancer cells A549 treated with TGF-β, showed decreased expression and activity of G6PD suggesting that the increased glucose uptaken by these cells is not being diverted to PPP (Lucena and Carvalho-Cruz et al. 2016). In the same model, glucose actually seems to be used in the HBP and cells display changes in cell surface glycosylation (Lucena and Carvalho-Cruz et al. 2016). HBP comprises the series of metabolic reactions that produce UDP-N-acetylglucosamine (UDP-GlcNAC), which is a substrate for O-, N-glycosylation and O-GlcNAcylation (Pinho and Reis 2015). UDP-GlcNAC is indeed increased in A549 cells treated with TGF-β, as well as several enzymes important for its formation, namely UDP-glucose pyrophosphorylase 2 (UGP2) and glutamine-fructose-6-phosphate transaminase 1 (GFPT1) together with proteins responsible for the N-linked glycans biosynthesis. The product of the GFPT1 gene is the rate enzyme of HBP, which is not only upregulated, but also shows increases activity after TGF-β treatment. Furthermore, O-linked β-N-acetyl glucosamine (O-GlcNAc) levels were also increased, while the enzymes that add or remove it from target proteins, were shown to regulate the phenotype of cells, namely the mesenchymal morphology, EMT markers and migration (Lucena and Carvalho-Cruz et al. 2016). The study by Alisson-Silva *et al.* showed another interesting link between the HBP, EMT and TGF-β (Alisson-Silva et al. 2013). A549 cells cultured in high glucose conditions showed N-cadherin and vimentin upregulation, mesenchymal morphology, increased motility concomitantly with increased TGF-β secretion. Besides, hyperglycemia induces an upregulation of fibronectin and the synthesis of the fibronectin splicing isoform that undergoes the addition of

an O-glycan to the peptide, oncofetal fibronectin (onfFN) (Alisson-Silva et al. 2013). This fibronectin isoform got its name from being almost exclusively associated with fetal tissue or related fluids, but it can also be detected in tumor cells and it has been suggested as a clinical biomarker in several cancer types (Alías-Melgar et al. 2013; Inufusa et al. 1995; Matsuura and Hakomori 1985; Mhawech et al. 2005). Similarly to what happens in lung cancer models (Alisson-Silva et al. 2013), TGF-β treatment of prostate epithelial cell lines induces upregulation of oncofetal fibronectin and total fibronectin, together with N-cadherin upregulation, E-cadherin downregulation and increased motility (Freire-de-Lima et al. 2011), being these alteration reversed by the knock-down of the enzymes that are involved in oncofetal fibronectin synthesis (Freire-de-Lima et al. 2011; Wandall et al. 2007).

The mitochondrial metabolic reprogramming driven by TGF-β in the context of EMT seems to be dependent on the cell type. In a study by Jiang *et al*. using NSCLC A549 cells, TGF-β treatment causes a higher oxygen consumption (Jiang et al. 2015), while Snail overexpression triggered similar effects in mitochondrial respiration. Another group of researchers showed that in several lung cancer cell lines with distinct genetic backgrounds - either KRAS or EGFR driven - treatment with TGF-β results in a decreased glycolysis to OXPHOS ratio (measured by proton production rate and oxygen consumption rate) (Sun et al. 2014). When becoming mesenchymal, these NSCLC cells show increased TCA cycle and OXPHOS activity. Moreover, the expression of the PDH-regulating kinase, PDK4, is reduced in NSCLC cells after treatment with TGF-β; if PDK4 overexpression partially inhibits TGF-β-driven EMT, on the other hand its knock-down in A549 and HCC827 cells is sufficient to promote vimentin and Zeb1 expression (Sun et al. 2014). These cells also showed a larger contribution of glucose to the TCA cycle intermediates and glutamate (Sun et al. 2014). The same authors also observed PDK4 downregulation upon TGF-β treatment both in human mammary epithelial MCF10A cells and in the same cells transfected with T24 c-Ha-Ras oncogene (MCF10AT) (Sun et al. 2014). The induction of EMT with TGF-β treatment in MCF10A neo T cells lowers the basal ROS levels (measured with DCFDA) (Farris et al. 2016). On the other hand, the NCI-H358 NSCLC cell line, after TGF-β treatment, shows a lower respiratory capacity, which can be rescued by the addition of pyruvate (Ulanet et al. 2014). These authors treated cells with the isoform 3 of TGF-β (TGF-β3), which may be behind the differences in metabolism. Despite the fact that all the TGF-β ligands have been shown to bind the type II receptors, their expression varies spatially and temporally and they may have distinct functions according to mice knock-out data (Akhurst and Hata 2012). In the case of EMT, a distinct role for the 3 isoforms of TGF-β had, in fact, already been suggested during development (Boyer et al. 1999; Brunet, Sharpe, and Ferguson 1995). More studies are needed to clarify each isoform is expressed during EMT in cancer of different organs and what are the effects on metabolism.

Intriguingly, TGF-β-induced EMT also results in different outcomes concerning oxygen consumption in pancreatic cancer cells, probably depending on the duration of the TGF-β treatment or the cell culture conditions, reflecting the extremely dynamic nature of metabolism. In Panc-1 cells oxygen consumption was shown to be decreased by Ji *et al.* (Ji et al. 2016), while Liu *et al.* reported no differences in oxygen consumption or in the amount of glucose that is oxidized to CO_2, which is supported by the absence of alterations in the expression of NDUFB8, SDHB and UQCRC2, enzymes that belong to the mitochondrial complexes I, II and III, respectively (Liu et al. 2016).

In MCF-7 cells, Lee *et al.* (2015) showed that TGF-β, which induces Snail upregulation, leads to a downregulation and a decrease in cytochrome c oxidase (COX) activity (or complex IV from the electron transport chain), which is reversed by Snail knock-down. These cells also consume less oxygen after TGF-β treatment (Lee et al. 2015). In another studied, it was shown in MDA-MB-231 and MCF-7 cells that Snail silencing by itself causes an increase in COX activity and oxygen consumption (Lee et al. 2012).

In CRC cells, loss of SDHB expression promoted EMT and cell migration and was associated with the expression of TGF-β pathway members, namely increased expression of SMAD3 and SMAD4 (in a way that can be rescued by SDHB expression) and upregulation of Snail in a TGFβ receptor 1- and SMAD4-dependent manner (Wang, Chen, and Wu 2016). When dividing CRC into SDHB high vs SDHB low (at the mRNA level), the tumors with low expression of SDHB tended to have higher levels of SMAD4, N-cadherin, Snail and lower levels of E-cadherin, when compared with the ones with high SDHB expression (Wang, Chen, and Wu 2016). SDH loss is associated with succinate accumulation and interestingly, succinate showed increased levels after Twist overexpression in mammary epithelial cells (Bhowmik et al. 2015). Not only SDH but also knock-down of FH, the enzyme that catalyzes the reaction after SDH in the TCA cycle, causes EMT with Twist and Slug upregulation (Sciacovelli et al. 2016). IDH mutations and the resulting 2-HG accumulation also induces EMT in a process mediated by ZEB1 (Colvin et al. 2016; Grassian et al. 2012).

TGF-β-induced EMT is also characterized by changes in the glutamine metabolism. Glutaminase is encoded by 2 genes - that encode for GLS1 and GLS2 isozymes - and each of these genes can give rise to two different isoforms (Aledo et al. 2000; Szeliga and Albrecht 2015). GSL1 and GLS2 show differential expression according to the tissue type (Aledo et al. 2000) and have been proposed to have different roles in cancer, although the mechanisms remain unknown. For example, GLS1 seems to be associated with oncogenic transformation and it is necessary for P493-6 human B lymphoma cells, PC3 human prostate cancer cells growth and its downregulation limits glioblastoma cell growth (Cheng et al. 2011; Gao et al. 2009), while GLS2 is a p53 gene

target and when overexpressed inhibits colony formation ability of several tumor cell lines (Hu et al. 2010). In MCF-7 cells, GLS1 was found to be upregulated after TGF-β treatment in a SMAD 2, 3 and 4 and distal-less homeobox-2 (Dlx-2) -dependent manner (Lee et al. 2016). Experiments with GLS1 knock-down and glutamine deprivation revealed that both GLS1 and glutamine are essential for TGF-β-induced E-cadherin downregulation and Snail upregulation, although overexpression of Snail does not affect GLS1 expression. The effects glutamine absence can be, in part, attenuated by the addition of a permeable form of α-ketoglutarate (Lee et al. 2016). In addition to regulating Snail expression, GLS1 also regulates Snail-induced metabolic phenotype. Snail overexpression increased glucose and lactate uptake, while decreasing oxygen consumption, in a process that could be reverted by GLS1 knockdown (Lee et al. 2016).

Glutamine anaplerosis of the TCA cycle was demonstrated in HCC cell lines, in a TGF-β context. HCC cells that show mesenchymal features, namely TGF-β and vimentin expression, in addition to E-cadherin downregulation, are more sensitive to glutamine depletion from the culture media (Soukupova et al. 2017). These cells show a higher contribution of glutamine to glutamate and to TCA cycle which is supported by a GLS1 upregulation and glutamine synthase downregulation, alongside a reduced mitochondrial respiration (Soukupova et al. 2017). Besides, inhibition of GLS1 impacts cell migration and inhibition of the receptor I for TGF-β results in GLS1 and glutamine transporter SLC7A5 downregulation (Soukupova et al. 2017).

Interestingly, when analyzing a panel of NSCLC cell lines according to their sensitivity to the GLS1 inhibitor BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide), low E-cadherin/high vimentin phenotype was positively correlated with enhanced cell sensitivity to the presence of BPTES (Ulanet et al. 2014). The same was observed after inducing EMT with TGF-β3 in the NSCLC NCI-H358 cell line, suggesting a link between increased GLS dependence and the EMT phenotype. In these mesenchymal cells, there is a differential expression balance of GLS1 isoforms, namely an increased GAC:KGA ratio (Ulanet et al. 2014). These GLS1 isoforms have different subcellular localizations in several tumor cell lines, with KGA localized in the cytosol but not in the mitochondria, while GAC is the only isoform present in the mitochondria (Cassago et al. 2012). This increased GAC:KGA ratio was also seen in primary NSCLC tumors and caused by a significant decrease of KGA expression (van den Heuvel et al. 2012). Moreover, this study suggests that the increased sensitivity to GLS1 inhibition of mesenchymal cells may be associated with a lower ability to adapt to other carbon sources to replenish the TCA cycle, resulting in a lower respiratory capacity and a decreased ability to control redox stress (Ulanet et al. 2014).

In NSCLC, TGF-β coordinates the expression of enzymes that control fatty acid synthesis from glucose (Jiang et al. 2015), for example by downregulating of FASN in A549 cells (Jiang et al.

2015). How can the modulation of lipids be important for these mesenchymal cells? In NSCLC cells, FASN knock-down itself (which also leads to a decreased contribution of glucose carbon to fatty acid synthesis) is able to increase motility, lung colonization *in vivo* and induce upregulation of vimentin and N-cadherin and downregulation of E-cadherin. The authors suggest that this type of metabolism may increase the availability of substrates for OXPHOS and favor energy production (Jiang et al. 2015). On the other hand, a positive feedback loop between TGF-β and FASN expression was shown in NSCLC cisplatin-resistant cells, which acquire EMT features in a TGF-β1 and FASN dependent manner (Yang et al. 2016). This was not observed in non-resistant cells (Yang et al. 2016), in accordance with the results from Jiang *et al.* (Jiang et al. 2015). Cisplatin-resistance associated EMT was characterized by increased levels of fatty acids and a strong upregulation of FASN, which, in turn, induces and is necessary for high TGF-β expression levels in cisplatin-resistant cells (Yang et al. 2016). The aforementioned studies show a tight regulation of FASN by TGF-β, which may be dependent on the alterations that are related with the acquisition of resistance. Indeed, EMT was shown to be important for chemoresistance in pancreatic and breast cancers (Fischer et al. 2015; Zheng et al. 2015). These results highlight the importance of the cellular context for the metabolic adaptations in cancer, which are highly dynamic and may play a crucial role in chemoresistance.

In HCC cell lines, an increase in fatty acid synthesis is concomitant with a decrease in fatty acid oxidation. In fact, HCC cells *in vitro* show increased expression of TGF-β and vimentin, together with decreased E-cadherin expression, in association with a downregulation in fatty acid β-oxidation enzymes and with upregulation of fatty acid synthesis enzymes and the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ). Accordingly, the cells associated with mesenchymal proteins show higher levels of sphingolipids and phospholipids, which is reversed by TGF-β receptor I inhibition (Soukupova et al. 2017). This suggests that the autocrine TGF-β signaling in HCC cells promotes lipid synthesis.

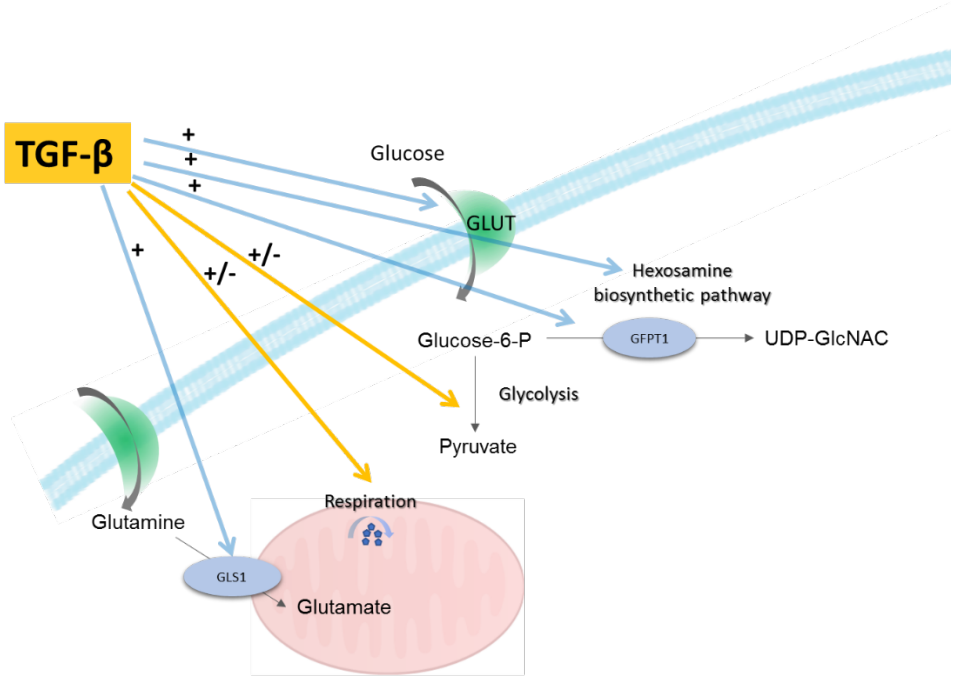

Fig. 1: The TGF-β pathway is tightly connected with metabolism. TGF-β treatment usually leads to increased glucose uptake through GLUTs. TGF-β has an important impact on two crucial metabolic processes – glycolysis and respiration – although the effects (positive or negative) are largely dependent on the tissue type. Glutamine metabolism and hexosamine biosynthesis are activated by TGF-β, through upregulation of GLS1 and GFPT1, respectively. (+) represents positive regulation; (-) represents negative regulation and (+/-) represents positive or negative regulation (both have been described). TGF-β, transforming growth factor β; GLUT, glucose transporter; glucose-6-P, glucose-6-phosphate; UDP-GlcNAc, UDP-N-acetylglucosamine; GLS1, glutaminase 1; GFPT1, glutamine-fructose-6-phosphate transaminase 1.

WNT

The Wnt pathway consists on the signaling cascade downstream of the Wnt ligands. Wnt1 was identified as Int-1 by Nusse and Varmus as a proto-oncogene that becomes activated upon integration of mouse mammary tumor virus (MMTV) proviral DNA which originates breast tumors (Nusse and Varmus 1982); subsequently, Wnt1 was found to be an homolog of the *Wingless* gene of *Drosophila*, a gene important for patterning decisions during embryonic development (Rijsewijk et al. 1987). Wnt ligands are secreted glycolipoproteins that act through autocrine and paracrine mechanisms to influence the fate of many cell types, either during embryonic development or in adult tissues (Van Camp et al. 2014; Dorsky, Moon, and Raible 1998). The binding of Wnt ligands to their cognate receptors, namely Frizzled receptor and LDL receptor family coreceptors, triggers the activation of a signaling cascade (MacDonald, Tamai,

and He 2018). The Wnt signaling is commonly divided into two pathways: the Wnt/ß-catenin or canonical pathway (the focus of this review) and the Wnt noncanonical pathway that includes the β-catenin-independent signaling (Zhan, Rindtorff, and Boutros 2017). In the canonical pathway, Wnt transduces downstream signals by stabilizing β-catenin, allowing its accumulation and subsequent translocation to the nucleus, where it forms a complex with TCF (T-cell-specific transcription factor)/LEF (lymphoid enhancer-binding factor) family of transcription factors and induces the expression of specific target genes. In unstimulated cells, cytosolic β-catenin is constitutively degraded by a ubiquitin ligase-proteosome system, in a process that is regulated by a complex of proteins such as axin, GSK-3β, casein kinase 1 and APC (Zhan, Rindtorff, and Boutros 2017).

Aberrant activation of the Wnt signaling pathway is associated with tumor development and/or progression, particularly in CRC (Bienz and Clevers 2000). The Wnt pathway involves important regulatory elements, whose genes are commonly dysregulated in cancer, often showing genetic and epigenetic alterations (Zhan, Rindtorff, and Boutros 2017). Of particular relevance are the *APC* mutations since they are the underlying cause of familial adenomatous polyposis, an hereditary colon cancer syndrome (Kinzler et al. 1991; Nishisho et al. 1991). Moreover, Wnt signaling has been described as affecting the maintenance of cancer stem cells and metastasis in breast cancer (Jang et al. 2015).

The Wnt signaling pathway incorporates and regulates several molecules that are important in mediating EMT or in defining the epithelial and mesenchymal phenotypes. In fact, β-catenin regulates the expression of mesenchymal proteins, such as fibronectin (Gradl, Kühl, and Wedlich 1999), metalloprotease 7 (Crawford et al. 1999) and vimentin (Gilles et al. 2003), the latter being able to promote β-catenin/TCF transcriptional activity (Satelli et al. 2016). On the other hand, β-catenin can also interact with E-cadherin cytoplasmic domain to maintain cell-cell adhesion. The balance between E-cadherin-bound and cytoplasmic-accumulated β-catenin is regulated by a series of kinases and phosphatases. It has been suggested, therefore, that E-cadherin and Wnt signaling can regulate each other through the pool of β-catenin (Nelson and Nusse 2004), although some authors argue against the regulation of Wnt signaling by E-cadherin (Vargas et al. 2016). Interestingly, N-glycosylation mediates the crosstalk between E-cadherin and the Wnt signaling: Wnt/β-catenin signaling regulates the expression of DPAGT1 (dolichyl-phosphate N-acetylglucosaminephosphotransferase 1), an enzyme that participates in the synthesis of glycoproteins such as E-cadherin and, consequently, modulates the function of adherens junctions (Sengupta, Bouchie, and Kukuruzinska 2010). N-glycosylation and the HBP are also involved in the activation of the Wnt signaling. Indeed, in macrophages, glucose leads to an increase in β-catenin protein levels and promotes β-catenin TCF/LEF family-dependent transcriptional events,

in a process that requires the HBP and N-glycosylation, possibly through an autocrine activation of the Wnt signaling system (Anagnostou and Shepherd 2008).

Glycosylation is not the only post-translational modification that depends on cellular metabolism: acetylation, for example, is regulated by the concentration of metabolic fuels (Zhao et al. 2010). Interestingly, acetylation of β-catenin was shown to regulate Wnt signaling: in conditions of Wnt stimulation, high levels of glucose promote the nuclear accumulation of acetylated β-catenin, by supporting the formation of LEF-1/β-catenin complex; the latter associates with the acetylase p300 and displaces the SIRT1 deacetylase, resulting in increased transcriptional activity (Chocarro-Calvo et al. 2013).

GSK-3, which takes part in the β-catenin destruction complex, is another component of the Wnt signaling that is regulated by a metabolic enzyme, namely the mitochondrial enzyme SDHF2, an assembly factor for SDH complex of the ETC. Knock-down of SDHF2 in lung cancer cells induces EMT and leads to an increase in β-catenin transcriptional activity. Particularly, this metabolic enzyme leads to an increase in GSK-3β activity, by decreasing the phosphorylation levels of serine 9, a negative regulatory site, in a process mediated by protein phosphatase 2A, resulting in decreased β-catenin levels and β-catenin/TCF transcriptional activity (Liu et al. 2013). GSK-3β is a serine-threonine kinase that, besides integrating the Wnt pathway, regulates cellular and body metabolism (Chen et al. 2016; Jellusova et al. 2017). In fact, GSK-3β was first identified as a regulator of glycogen synthase, a key enzyme in glycogen synthesis (Embi, Rylatt, and Cohen 1980). GSK-3β phosphorylates and inhibits glycogen synthase, having an inhibitory effect on glycogen synthesis (Eldar-Finkelman et al. 1996).

Another interesting link between cellular metabolism, Wnt pathway and EMT is through hypoxia. Although in some contexts hypoxia inhibits Wnt proliferative effects through several mechanisms (Kaidi, Williams, and Paraskeva 2007; Lim, Chun, and Park 2008; Verras et al. 2008), in conditions where hypoxia induces EMT, β-catenin plays an important role in mediating the downregulation of epithelial proteins, upregulation of mesenchymal proteins and EMT TFs, as well as in the acquisition of migratory features. Wnt/β-catenin can actually enhance hypoxia-induced EMT (Liu et al. 2010; Zhang et al. 2013; Zhao et al. 2011).

Wnt ligands have been shown to trigger cancer-associated EMT. For example, Wnt-1 and Wnt-3 overexpression leads to E-cadherin downregulation and to an invasive phenotype (Qi et al. 2014; Yook et al. 2005), whilst Wnt signaling also increases the protein level of several EMT TFs, such as Snail, Slug, Twist, Zeb (Howe et al. 2003; Qi et al. 2014; Sánchez-Tilló et al. 2011; Wu et al. 2012; Yook et al. 2005). In the case of Snail, Wnt signaling leads to its upregulation by enhancing protein stabilization: in the absence of Wnt signaling, Snail can be phosphorylated by GSK-3β, leading to its ubiquitination and proteosomal degradation. Conversely, active Wnt signaling

induces Axin2 expression, in a process mediated by β-catenin–TCF complex. Axin2 acts as a nucleocytoplasmic chaperone for GSK-3β, promoting Snail stabilization (Yook et al. 2005, 2006; Zhou et al. 2004). Wnt-triggered EMT, either by Wnt1 overexpression or Wnt3 ligand treatment is characterized by metabolic changes in MCF-7 cells, which cells show an increased glucose uptake (Lee et al. 2012, 2016) and a decreased oxygen consumption (Lee et al. 2012, 2016). Previously, a gene expression analysis in livers from transgenic mice overexpressing liver-specific β-catenin had also identified pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase as among the upregulated genes (Tan et al. 2005). Pate *et al.* also showed that CRC cells exhibit decreased glycolysis after Wnt signaling inhibition (Pate et al. 2014).

Cells undergoing EMT upon Wnt1 overexpression or Wnt3 treatment show decreased oxygen consumption together with a reduction in COX activity and diminished transcriptional activity of its subunits, in a process possibly mediated by Snail binding to its promoter (Lee et al. 2012). Another study, using MDA-MB-231 cells (a triple negative breast cancer cell line), showed that Wnt modulates mitochondrial homeostasis since Wnt5B knock-down not only impairs cell motility but also results in mitochondrial morphological alterations, decreased mtDNA content and a downregulation of several mitochondrial proteins, namely mitochondrial import receptor TOMM20 (translocase of outer mitochondrial membrane 20), cytochrome c1 and the subunit of mitochondrial ATP synthase ATP5MC1 (ATP synthase membrane subunit c locus 1) (Yang et al. 2014). Concerning lactate production, two studies showed different results, probably related with the cell culture conditions: Lee *et al*. reported an increased lactate production after Wnt signaling activation (Lee et al. 2012) while Kim *et al.* observed a decreased production of lactate (Kim et al. 2017). A third study showed that blocking Wnt signaling, through overexpression of dominant negative LEF/TCF isoforms that lack the β-catenin binding domain, results in a decrease lactate production in CRC cells (Pate et al. 2014). Interestingly, this Wnt signaling reduction leads to downregulation of PDK1 and MCT1, both being direct Wnt target genes (Pate et al. 2014).

Additionally, GLS1 is upregulated upon Wnt-induced EMT, in a β-catenin, axin, TCF4 dependent and Snail independent manner (Lee et al. 2016). Experiments with GLS1 knock-down and glutamine deprivation revealed that GLS1 is essential for Wnt-induced mesenchymal morphology and E-cadherin downregulation (Lee et al. 2016). Besides, the increased glucose consumption, lactate production and decreased oxygen consumption in this system are dependent on GLS1 expression (Lee et al. 2016).

Myc is a target gene of the Wnt pathway (He et al. 1998; Sansom et al. 2007; van de Wetering et al. 2002) that is at the cross-roads of EMT and metabolism. Myc has been associated with EMT and it is an important regulator of cancer cell metabolism, with effects on glycolysis and glutamine metabolism (Goetzman and Prochownik 2018). The oncogene c-Myc stimulates

glutamine utilization through the coordinate transcription of genes necessary for cells to engage in glutamine catabolism (Wise et al. 2008). For example, c-Myc can upregulate GLS by regulating miR-23a and miR-23b, being glutamine and GLS required for Myc-mediated cancer cell proliferation and survival (Gao et al. 2009). The translation efficiency of c-Myc is, in turn, regulated by mTOR/S6K1 signaling, via eIF4B, leading to a mTOR-dependent control of the glutamine flux through regulation of GLS (Csibi et al. 2014). Myc has also been implicated in the regulation of other metabolic enzymes like LDHA (Shim et al. 1997). Although the role of c-Myc in EMT is still underexplored, there is some evidence that its overexpression in mammary epithelial cells (MCF10A and IMECs) and in transformed human bronchial epithelial cells leads to downregulation of E-cadherin and upregulation of mesenchymal associated proteins such as N-cadherin, vimentin, Snail and Zeb1/2 (Cho et al. 2010; Cowling and Cole 2006; Larsen et al. 2016). Myc is required for an effective induction of EMT by TGF-β, namely E-cadherin downregulation, E-cadherin and β-catenin decrease at the cell surface and fibronectin upregulation in breast epithelial cells and prostate cancer cells (Amatangelo et al. 2012; Smith et al. 2008). In the particular case of mammary epithelial cells and breast cancer cells (MDA-MB-231 cells), Myc binds to the Snail promoter, facilitating a rapid Snail upregulation upon TGF- β treatment (Smith et al. 2008). Also in MDA-MB-231 cells, Myc has been proposed to mediate the role of Wnt signaling in the maintenance of mitochondrial physiology (Yang et al. 2014).

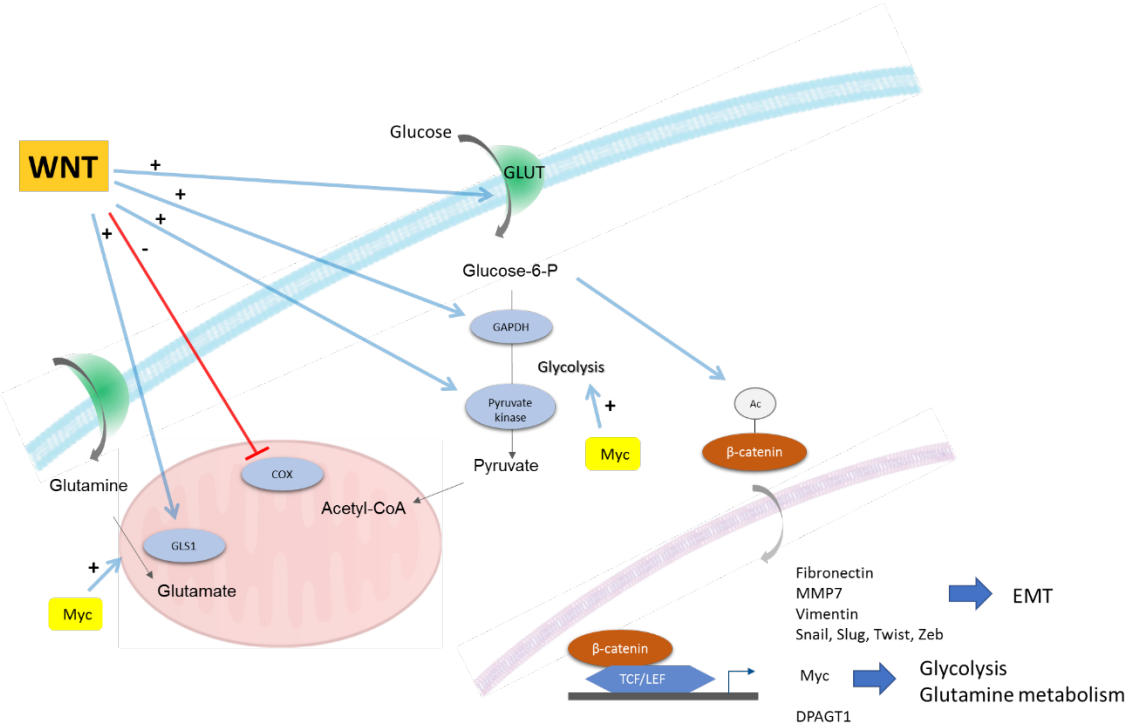

Fig. 2: The WNT pathway has been shown to regulate metabolism during EMT, namely mitochondrial metabolism – glutamate synthesis through GLS1 or OXPHOS through COX - and glycolysis, either directly or through Myc. GLUT, glucose transporter; glucose-6-P, glucose-6-

phosphate; GLS1, glutaminase 1; COX, cytochrome c oxidase; TCF/LEF, T-cell-specific transcription factor / lymphoid enhancer-binding factor; DPAGT1, dolichyl-phosphate N-acetylglucosaminephosphotransferase 1; Zeb, zinc-finger E-box-binding.

HIPPO

The Hippo pathway was first characterized in *Drosophila* and it is named after the *hpo* gene, which encodes a kinase involved in tissue growth restriction in this model organism (reviewed in Pan 2007). The Hippo pathway can be triggered and regulated by several extracellular cues like adhesion between cells, interactions with the matrix and extracellular ligands, playing important roles in cell proliferation, growth, differentiation and organ growth (Hong et al. 2005; Kim and Gumbiner 2015; Park et al. 2015; Yu et al. 2012; Zhao et al. 2007, 2012; Zhou et al. 2015). In mammals, the activity of the core Hippo pathway is mediated by several kinases, namely the mammalian Ste20-like kinases (MST) 1, 2 and the large tumor suppressor (LATS) 1, 2 kinases, adaptor proteins, downstream effectors - Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) - and nuclear transcription factors (being TEAD family of transcription factors the main transcriptional output) (Meng, Moroishi, and Guan 2016). TAZ and YAP are two main effectors of the Hippo pathway in mammals and work as transcription co-activators, regulating the expression of a variety of genes important for cellular proliferation, apoptosis and differentiation (Dong et al. 2007; Hong et al. 2005). The Hippo pathway inhibits YAP/TAZ activity, in a process mediated by TAZ phosphorylation by MST and LATS, which results in YAP/TAZ cytoplasmic retention (Lei et al. 2008; Zhao et al. 2007). TAZ/YAP can also be regulated independently of Hippo pathway core kinases, for example by activation of the actomyosin cytoskeleton and Rho GTPase upon mechanical signals exerted by extracellular matrix rigidity and cell shape (Dupont and Morsutet al. 2011). Importantly, the Hippo pathway effectors YAP/TAZ also respond to metabolic cues. Energetic stress, such as ETC inhibition, glucose withdrawal and inhibition of glucose metabolism by 2-DG, leads to phosphorylation and consequent inhibition of YAP activity, in a process mediated by AMPK (DeRan et al. 2014; Wang et al. 2015). Not only phosphorylation, but also other post-translational modifications can modulate YAP/TAZ activity, including SET-7-mediated methylation (Oudhoff et al. 2018) and acetylation by CREB binding protein and p300 (Hata et al. 2012). The substrates for these reactions are the products of several metabolic pathways and therefore, the availability of metabolic fuels and the cellular metabolism may regulate Hippo pathway (Filipp 2017; Zhao et al. 2010).

The Hippo pathway is tightly associated with epithelial cell polarity and cell junctions, both of which are lost during EMT (Martin-Belmonte and Perez-Moreno 2011). In fact, epithelial cell

polarity suppresses YAP/TAZ activity through the Crumbs polarity complex, while interference with the Crumbs complex in epithelial cells activates YAP/TAZ, which in turn promotes SMAD nuclear accumulation, TGF-β signaling and EMT (Varelas et al. 2010). Scribble, another cell polarity protein localized in the cell membrane, also inhibits TAZ by regulating the Hippo pathway (Cordenonsi et al. 2011). In MCF10A breast epithelial cells, the overexpression of a constitutively active form of TAZ actually results in a mesenchymal morphology, together with E-cadherin and occludin downregulation and the upregulation of N-cadherin, vimentin and fibronectin (Lei et al. 2008). Overexpression of active TAZ also confers cells increased migratory properties and proliferation (Lei et al. 2008). As an important regulator of stem cell differentiation and renewal, the Hippo pathway may have an active role in the context of cancer, where EMT has been associated with stem-like features (Bae et al. 2015; Sun et al. 2014). In MCF10A cells overexpressing HRas, TAZ not only results in decreased E-cadherin expression and increased migration but also increases soft-agar colony formation and mammosphere formation capacity (Cordenonsi et al. 2011; Mulvihill et al. 2014). In this system, TAZ overexpression is associated with changes in the expression of several metabolic enzymes and in the abundance of metabolites (Mulvihill et al. 2014). Particularly, in cells that were selected to mimic cellular transformation and malignant progression of breast cancer (through HRAS transfection, selection for spontaneous progression *in vivo* and overexpression of TAZ), FASN, platelet activating factor acetylhydrolase (PAFAH) 1B2 and PAFAH1B3 (two different lipases) were found to be upregulated and dysregulated in their serine hydrolase-directed activity. PAFAH1B is essential for the maintenance of aggressive features in triple-negative breast cancer cells, which is possibly mediated by alterations in the lipid landscape (Mulvihill et al. 2014).

Other studies have also showed a link between metabolism and TAZ/YAP. For example, YAP induces GLUT3 expression to promote glucose uptake, and YAP and GLUT3 expression positively correlated in human liver and colon cancer (Wang et al. 2015). In TNBC cells, YAP induces hexokinase (HK) 2 and PFKB3 expression, leading to increased glycolysis; this metabolic effect is mediated by the lncRNA, breast cancer anti-estrogen resistance 4 (BCAR4) and the Hedgehog signaling, which are required for TNBC metastasis.

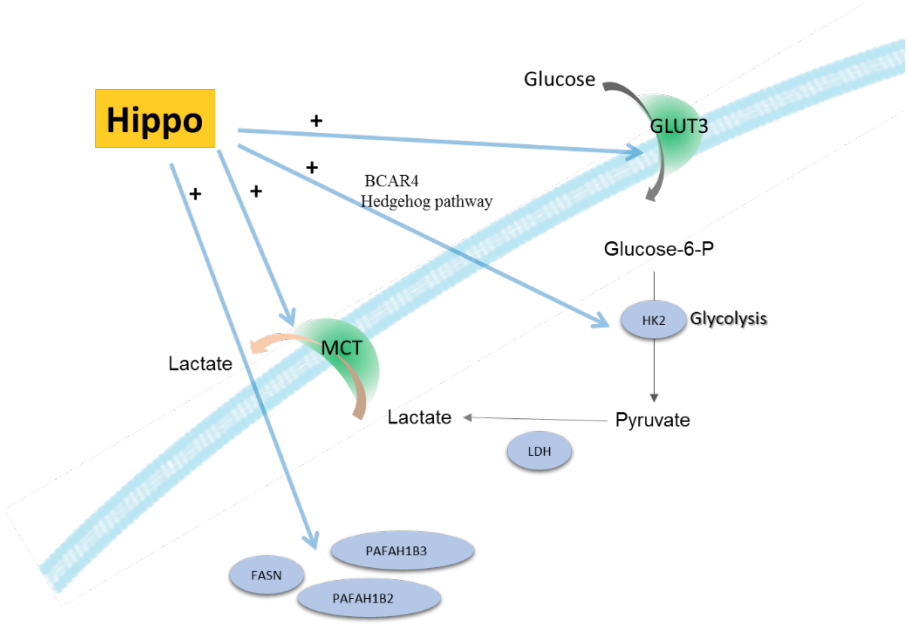

Fig. 3: In the Hippo pathway there is upregulation of GLUT3 and HK2 to increase glucose uptake and catabolism, respectively. Increased glycolysis seems to be mediated by BCAR4 and the Hedgehog pathway. There is also a close relation with lipid metabolism, namely upregulation of FASN, PAFAH1B2 and PAFAH1B3. GLUT3, glucose transporter 3; glucose-6-P, glucose 6 phosphate; HK2 hexokinase 2, LDH, lactate dehydrogenase; PAFAH, platelet activating factor acetylhydrolase; FASN, fatty acid synthase; BCAR4, breast cancer anti-estrogen resistance 4.

CONCLUSIONS

The interplay between metabolism and metastasis is an emerging area of research. Some studies are particularly focused on EMT, a transdifferentiation program associated with metastasis, where the rewiring of several metabolic pathways appears as an important phenomenon. Noteworthy, some of the studies described in this manuscript show that metabolism is not only a consequence of changes in the cellular phenotype, rather playing an active role in EMT induction and maintenance. However, few studies explore the mechanism by which metabolism regulates EMT. Likewise, there is little data distinguishing the metabolic changes that are specifically related with the transition itself from those that are required for the maintenance of a mesenchymal phenotype.

The majority of the EMT models display changes in metabolism towards an increased glycolysis, characterized by enhanced glucose uptake and lactate production, showing also alterations in OXPHOS activity. These metabolic shifts are supported by alterations in the expression or activity of metabolic enzymes, through mechanisms that involve, for example, direct binding of Snail to

the gene promoter. Moreover, the TCA cycle, glutamine and lipid metabolism might also play important roles for the acquisition of mesenchymal properties.

It should be pointed out that the link between EMT and metabolism needs to be integrated in the cellular context. In cancer cells, the uptake of nutrients is controlled not only by growth factor signaling but also by the aberrantly activated signaling pathways as consequence of their oncogenic alterations (Hanahan and Weinberg 2000). Indeed, the reprogramming of tumor metabolism has been shown to be under the control of various oncogenic signals (Edinger and Thompson 2002; Levine and Puzio-Kuter 2010; Thompson 2011). Major signaling pathways associated with cancer progression, such as TGF-β, Wnt and Hippo play a key role in the acquisition of mesenchymal characteristics in several cancer types. They regulate several players, in a complex network, that, in the case of EMT, includes the upregulation of EMT TFs. Indeed, Bhowmik *et al.* showed that the differential expression of EMT TFs results in distinctive metabolic profiles, although with some common alterations (Bhowmik et al. 2015). Nonetheless, a deeper analysis of the signaling landscape and its role in mediating the metabolic alterations in the context of EMT is lacking. The data is still limiting for the signaling pathways analyzed in this review and even more for other key signaling cascades like the Ras-MAPK. Besides, there is also a need of studies that analyze EMT and metabolic changes in a temporal manner, in order to distinguish early changes from later ones.

In this review, we could further conclude that, even when evaluating the metabolism of EMT under the perspective of specific signaling pathways there is still heterogeneity. One might ask: why is there so much diversity in the metabolic reprogramming that characterizes EMT? Metabolism is a highly dynamic and plastic process, probably reflecting the tissue of origin and the genetic alterations that characterize the cells models, which go from nontransformed cells to cancer-derived cells, most commonly from the primary tumor but in some cases from metastatic sites. This heterogeneity conceivably translates what happens *in vivo*, especially because tumor cells are exposed to multiple signals from the microenvironment, adding another layer of complexity.

Although this review focused on the literature about cancer-associated EMT, metabolism likely plays an important role in EMT during embryonic development and fibrosis. Different metabolic profiles have been reported in specific phases of embryonic development and metabolism plays a key role in the function of embryonic stem cells (Pereira et al. 2014) However, there is still a very limited number of studies addressing metabolism during the EMT that occurs in embryonic development and fibrosis.

Targeting metabolism in combination with anti-neoplastic drugs holds promise as a strategy to disrupt the formation of metastasis. The study of metabolism during EMT may expose metabolic

vulnerabilities that can be explored as therapeutic targets for cancer metastasis. Reversing EMT by targeting cellular metabolism might for example, contribute to drug sensitivity. Besides, the metabolic players in EMT can become promising new biomarkers of cancer progression.

It is important to note that TGF-β, Wnt/β-catenin and Hippo pathways are currently being explored as therapeutic targets (Akhurst 2017; Johnson and Halder 2014; Krishnamurthy and Kurzrock 2018), so it is imperative to understand the crosstalk between signaling and metabolic networks during EMT.

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